METHODS OF GENERATING HIGH-PRODUCTION OF ANTIBODIES FROM HYBRIDOMAS CREATED BY *IN VITRO* IMMUNIZATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This Application claims the benefit of U.S. Provisional Application No. 60/427,165 filed November 15, 2002 and U.S. Provisional Application No. 60/501,650 filed September 10, 2003, the disclosures of which are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

[0002] The invention relates to the generation of hybridoma cells that produce high-affinity antibodies in high titers. More specifically, the invention relates to the use of an *in vitro* immunization method in conjunction with hybridoma technology using dominant negative mismatch repair genes or chemical inhibitors of mismatch repair to produce high titers of antigen specific antibodies of the IgG subclass, that bind to the antigen with high affinity.

BACKGROUND OF THE RELATED ART

[0003] The use of antibodies to block the activity of foreign and/or endogenous polypeptides provides an effective and selective strategy for treating the underlying cause of disease. In particular is the use of monoclonal antibodies (MAb) as effective therapeutics such as theFDA approved ReoPro (Glaser, (1996) *Nat. Biotechnol.* 14:1216-1217), an anti-platelet MAb from Centocor; Herceptin (Weiner, (1999) *Semin. Oncol.* 26:43-51), an anti-Her2/neu MAb from Genentech; and Synagis (SaezLlorens, *et al.* (1998) *Pediat. Infect. Dis. J.* 17:787-791), an anti-respiratory syncytial virus MAb produced by Medimmune.

[0004] Standard methods for generating MAbs against candidate protein targets are known by those skilled in the art. Briefly, rodents such as mice or rats are injected with a purified antigen in the presence of adjuvant to generate an immune response (Shield, *et al.* (1996) *Am. J. Kidney Dis.* 27: 855-864). Rodents with positive immune sera are sacrificed and splenocytes are isolated. Isolated splenocytes are fused to melanomas to produce immortalized cell lines that are then screened for antibody production. Positive lines are

isolated and characterized for antibody production. The direct use of rodent MAbs as human therapeutic agents were confounded by the fact that human anti-rodent antibody (HARA) responses occurred in a significant number of patients treated with the rodent-derived antibody (Khazaeli, *et al.*, (1994) *Immunother.* 15:42-52). In order to circumvent the problem of HARA, the grafting of the complementarity determining regions (CDRs), which are the critical motifs found within the heavy and light chain variable regions of the immunoglobulin (Ig) subunits making up the antigen binding domain, onto a human antibody backbone found these chimeric molecules are able to retain their binding activity to antigen while lacking the HARA response (Emery and Harris, "Strategies for humanizing antibodies" In: ANTIBODY ENGINEERING, C.A.K. Borrebaeck (Ed.) Oxford University Press, NY, 1995. pp. 159-183. A common problem that exists during the "humanization" of rodent-derived MAbs (referred to hereafter as HAb) is the loss of binding affinity due to conformational changes in the three-dimensional structure of the CDR domain upon grafting onto the human Ig backbone (U. S. Patent No. 5,530,101 to Queen *et al.*). To overcome this problem, additional HAb vectors are usually needed to be engineered by inserting or deleting additional amino acid residues within the framework region and/or within the CDR coding region itself in order to recreate high affinity HAbs (U. S. Patent No. 5,530,101 to Queen*et al.*). This process is a very time consuming procedure that involves the use of expensive computer modeling programs to predict changes that may lead to a high affinity HAb. In some instances the affinity of the HAb is never restored to that of the MAb, rendering them of little therapeutic use.

[0005] Another problem that exists in antibody engineering is the generation of stable, high yielding producer cell lines that is required for manufacturing of the molecule for clinical materials. Several strategies have been adopted in standard practice by those skilled in the art to circumvent this problem. One method is the use of Chinese Hamster Ovary (CHO) cells transfected with exogenous Ig fusion genes containing the grafted human light and heavy chains to produce whole antibodies or single chain antibodies, which are a chimeric molecule containing both light and heavy chains that form an antigen-binding polypeptide (Reff, M. E. (1993) *Curr. Opin. Biotechnol.* 4:573-576). Another method employs the use of human lymphocytes derived from transgenic mice containing a human grafted immune system or transgenic mice containing a human Ig gene repertoire. Yet another method employs the use of monkeys to produce primate MAbs, which have been reported to lack a human anti-monkey response (Neuberger and Gruggermann (1997) *Nature* 386:25-26). In all cases, the generation of a cell line that is capable of generating sufficient amounts of high affinity antibody poses a major limitation for producing sufficient materials for clinical studies. Because of these

limitations, the utility of other recombinant systems such as plants are currently being explored as systems that will lead to the stable, high-level production of humanized antibodies (Fiedler and Conrad (1995) *Bio/Technology* 13:1090-1093).

[0006] One strategy to overcome the problem of human reactions against foreign antibodies is to stimulate human immunoglobulin-producing cells *in vitro*. Various attempts to stimulate human antibody production *in vitro* typically have resulted in low affinity antibodies of the IgM subclass (Zafiropoulos *et al.* (1997) *J. Immunological Methods* 200:181-190).

[0007] A method for generating diverse antibody sequences within the variable domain that results in HAbs and MAbs with high binding affinities to antigens would be useful for the creation of more potent therapeutic and diagnostic reagents respectively. Moreover, the generation of randomly altered nucleotide and polypeptide residues throughout an entire antibody molecule will result in new reagents that are less antigenic and/or have beneficial pharmacokinetic properties. The invention described herein is directed to the use of random genetic mutation throughout an antibody structure *in vitro* by blocking the endogenous mismatch repair (MMR) activity of a host cell producing immunoglobulins that encode biochemically active antibodies. The invention also relates to methods for repeated *in vitro* genetic alterations and selection for antibodies with enhanced binding and pharmacokinetic profiles.

[0008] In addition, the ability to develop genetically altered host cells that are capable of secreting increased amounts of antibody also will provide a valuable method for creating cell hosts for product development. The invention described herein is further directed to the creation of genetically altered cell hosts with increased antibody production via the blockade of MMR. The invention facilitates the generation of high affinity antibodies and the production of cell lines with elevated levels of antibody production derived from hybridoma cells. The invention described herein provides methods for generating antigen-specific monoclonal antibodies (mAbs). Other advantages of the present invention are described in the examples and figures described herein.

SUMMARY OF THE INVENTION

[0009] The invention provides methods for producing hybridoma cells producing high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells, wherein the hybridoma cells express a dominant negative allele of a

mismatch repair gene; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; thereby producing hybridoma cells producing high-affinity antibodies.

[0010] In some embodiments, the dominant negative allele of a mismatch repair gene comprises a truncation mutation of the PMS2 gene (*e.g.*, a PMS2-134 gene). In some embodiments of the method of the invention, antibodies are screened using an ELISA-based assay or other assays that can measure antibody-antigen binding. In some embodiments, the screening assays screen for hypermutated hybridomas that produce higher affinity antibodies than those produced by the parental hybridomas. In other embodiments, the screening assays screen for hypermutated hybridomas that produce antibodies in higher titers than the parental hybridomas.

[0011] In some embodiments of the method of the invention, the method further comprises inactivation of the dominant negative allele of the mismatch repair gene, thereby stabilizing the genome of said hypermutated hybridoma.

[0012] In some embodiments of the method of the invention, the dominant negative mismatch repair gene is introduced into the hybridoma cell after the fusion of said myeloma with the immunoglobulin-producing cells. In other embodiments, the dominant negative mismatch repair gene is introduced into the myeloma cell prior to the fusion with the immunoglobulin-producing cells.

[0013] The invention also comprises antibodies produced by the hybridoma cells.

[0014] The invention also comprises methods for producing hybridoma cells that produce high titers of antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor blood cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells, wherein the hybridoma cells express a dominant negative allele of a mismatch repair gene; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen of the hypermutated hybridoma cells for antigen-specific antibodies produced in higher titers than that produced by the parental hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce higher titers of antibodies than that produced by the parental hybridoma cells.

[0015] In some embodiments, the dominant negative allele of a mismatch repair gene comprises a truncation mutation of the PMS2 gene (*e.g.*, a PMS2-134 gene). In some embodiments of the method of the invention, antibodies are screened using an ELISA-based assay. In some embodiments, the screening assays screen for hypermutated hybridomas that produce higher affinity antibodies than those produced by the parental hybridomas. In other embodiments, the screening assays screen for hypermutated hybridomas that produce antibodies in higher titers than the parental hybridomas.

[0016] In some embodiments of the method of the invention, the method further comprising inactivation of the dominant negative allele of the mismatch repair gene, thereby stabilizing the genome of said hypermutated hybridoma.

[0017] In some embodiments of the method of the invention, the dominant negative mismatch repair gene is introduced into the hybridoma cell after the fusion of said myeloma with the immunoglobulin-producing cells. In other embodiments, the dominant negative mismatch repair gene is introduced into the myeloma cell prior to the fusion with the immunoglobulin-producing cells.

[0018] In some embodiments of the method of the invention, the dominant negative allele of the mismatch repair gene is subsequently inactivated in order to restabilize the genome of the cell.

[0019] The dominant negative allele of the mismatch repair gene may be introduced into the myeloma cell prior to fusion with the immunoglobulin producing cells. Thus, the resulting hybridoma cells express the same dominant negative allele of the mismatch repair gene as the myeloma cells. Alternatively, the dominant negative allele of the mismatch repair gene may be introduced into the hybridoma cells.

[0020] The invention also comprises antibodies produced by the hybridoma cells.

[0021] The invention further provides recombinant myeloma cells comprising a polynucleotide sequence encoding a dominant negative mismatch repair protein. The dominant negative mismatch repair protein may be a dominant negative form of, for example, a PMS2, PMS1, PMSR3, PMSR2, PMSR6, MLH1, GTBP, MSH3, MSH2, MLH3, or MSH1 and PMSR proteins encoded by homologs of the *PMSR* genes as described in Nicolaides *et al.* (1995) *Genomics* 30:195-206 and Horii *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:1257-1264. In some embodiments, the recombinant myeloma cell expresses a polynucleotide encoding a dominant negative allele of a *PMS2* gene (*e.g.*, a truncation mutation of the PMS2 gene, such as the *PMS2-134* gene).

[0022] In some embodiments, the recombinant myeloma cell is a human cell. In other embodiments, the recombinant myeloma cell does not express immunoglobulin genes and/or Epstein-Barr virus. In other embodiments, the myeloma cells are HAT sensitive.

[0023] The invention also provides a method for producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing said immunoglobulin-producing cells with myeloma cells to form hybridoma cells; (c) performing a screen for binding of antibodies produced from said hybridoma cells to antigen; (d) cloning immunoglobulin genes from said hybridoma into a mammalian expression cell, wherein said mammalian expression cell expresses a dominant negative allele of a mismatch repair gene; (e) performing a screen for mammalian expression cells that secrete antibodies with higher affinity for antigen as compared to antibodies produced from said hybridoma cells; thereby producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0024] In some embodiments, the dominant negative allele of a mismatch repair gene is introduced into said mammalian expression cell prior to introduction of the immunoglobulin genes. In other embodiments, the dominant negative allele of a mismatch repair gene is introduced into said mammalian expression cell after introduction of said immunoglobulin genes. In other embodiments, the dominant negative allele of a mismatch repair gene is introduced into the mammalian expression cell with the immunoglobulin genes simultaneously.

[0025] The invention also comprises antibodies produced by the mammalian expression cells.

[0026] The invention also provides a method for producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing said immunoglobulin-producing cells with myeloma cells to form hybridoma cells, wherein said hybridoma cells express a dominant negative allele of a mismatch repair gene; (c) incubating said parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from said hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with greater affinity for said antigen than antibodies produced by said parental hybridoma cells; (f) cloning immunoglobulin genes from said hybridoma into a mammalian expression cell; thereby

producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized human immunoglobulin-producing cells.

[0027] In some embodiments, the dominant negative allele of a mismatch repair gene is present in the myeloma cell prior to cell fusion. In other embodiments, the dominant negative allele of the mismatch repair gene is introduced into the hybridoma cell after cell fusion.

[0028] The invention also comprises antibodies produced by the mammalian expression cells.

[0029] The invention also provides a method for producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form hybridoma cells; (c) performing a screen for binding of antibodies produced from the hybridoma cells to antigen; (d) cloning immunoglobulin genes from the hybridoma into a parental mammalian expression cell, wherein the mammalian expression cell expresses a dominant negative allele of a mismatch repair gene; (e) incubating the parental mammalian expression cell to allow for mutagenesis, thereby forming hypermutated mammalian expression cells; (f) performing a screen of hypermutable mammalian expression cells that secrete antibodies with higher affinity for antigen as compared to antibodies produced from the hybridoma cells; and (g) performing a screen of hypermutable mammalian expression cells that secrete higher titers of antibodies than parental mammalian expression cells; thereby producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0030] In some embodiments, the dominant negative allele of a mismatch repair gene is introduced into said mammalian expression cell prior to introduction of the immunoglobulin genes. In other embodiments, the dominant negative allele of a mismatch repair gene is introduced into said mammalian expression cell after introduction of said immunoglobulin genes. In other embodiments, the dominant negative allele of a mismatch repair gene is introduced into the mammalian expression cell with the immunoglobulin genes simultaneously.

[0031] The invention also provides antibodies produced by the mammalian expression cells.

[0032] The invention also provides recombinant, hypermutable mammalian expression cells comprising a polynucleotide sequence encoding a dominant negative mismatch repair protein.

[0033] The mismatch repair gene may be a dominant negative mismatch repair gene, including, but not limited to a dominant negative form of *PMS2*, *PMS1*, *PMSR3*, *PMSR2*, *PMSR6*, *MLH1*, *GTBP*, *MSH3*, *MSH2*, *MLH3*, or *MSH1*, and homologs of *PMSR* genes as

described in Nicolaides *et al.* (1995) *Genomics* 30:195-206 and Horii *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:1257-1264. A non-limiting example includes a dominant negative truncation mutant of *PMS2* (*e.g.*, a *PMS2-134* gene).

[0034] The invention also provides methods for producing hybridoma cells producing high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells; (c) incubating the parental hybridoma cells in the presence of at least one chemical inhibitor of mismatch repair, thereby forming hypermutated hybridoma cells; (d) performing a screen for antigen binding for antibodies produced from the hypermutated hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce antibodies with greater affinity for the antigen than antibodies produced by said parental hybridoma cells; thereby producing hybridoma cells producing high-affinity antibodies.

[0035] The invention also comprises antibodies produced by the hybridoma cells.

[0036] The invention also provides methods for producing hybridoma cells that produce high titers of antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells; (c) incubating the parental hybridoma cells in the presence of at least one chemical inhibitor of mismatch repair, thereby forming hypermutated hybridoma cells; (d) performing a screen of the hypermutated hybridoma cells for antigen-specific antibodies produced in higher titers than that produced by the parental hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce higher titers of antibodies than that produced by said parental hybridoma cells; thereby producing hybridoma cells producing high titers of antibodies.

[0037] In some embodiments of the method of the invention, the hypermutated hybridoma cells also are screened for the production of higher titers of antibodies than that produced by the parental hybridomas. The screening may be using an ELISA-based assay, or any other means to measure antibody-antigen binding.

[0038] The invention also comprises antibodies produced by the hybridoma cells.

[0039] The invention also provides methods for producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells

with myeloma cells to form hybridoma cells; (c) performing a screen for antigen binding of antibodies produced from the hybridoma cells; (d) cloning immunoglobulin genes from the hybridoma cells into a mammalian expression cell; (e) incubating the mammalian expression cell in the presence of at least one chemical inhibitor of mismatch repair; (f) performing a screen for mammalian expression cells that secrete antibodies with higher affinity for antigen as compared to antibodies produced from the hybridoma cells; thereby producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0040] In some embodiments of the method of the invention the method may further comprise the removal of the chemical inhibitor from the hypermutated mammalian expression cells, thereby stabilizing the genome of said hypermutated mammalian expression cells.

[0041] The invention also comprises antibodies produced by the mammalian expression cells

[0042] The invention also provides methods for producing mammalian expression cells that produce high titers of high affinity antibodies to a selected antigen from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form hybridoma cells; (c) incubating the hybridoma cells in the presence of at least one chemical inhibitor of mismatch repair to form hypermutated hybridoma cells; (d) performing a screen for antigen binding for antibodies produced from the hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with greater affinity for the antigen than antibodies produced by the parental hybridoma cells; (f) cloning immunoglobulin genes from the hypermutated hybridoma cells into a mammalian expression cell, thereby forming parental mammalian expression cells; thereby producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0043] In some embodiments, the parental mammalian expression cell is further incubated in the presence of at least one chemical inhibitor of mismatch repair, thereby forming a hypermutated mammalian expression cell; and the hypermutated mammalian expression cells are screened for higher production of antibodies than that of the parental mammalian expression cells.

[0044] In some embodiments of the method of the invention the method may further comprise the removal of the chemical inhibitor from the hypermutated hybridoma and/or hypermutated mammalian expression cells, thereby stabilizing the genome of said hypermutated hybridoma cells and/or hypermutated mammalian expression cells.

[0045] The invention also comprises antibodies produced by the mammalian expression cells.

[0046] The invention also provides a method for producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing said immunoglobulin-producing cells with myeloma cells to form hybridoma cells; (c) performing a screen for binding of antibodies produced from said hybridoma cells to antigen; (d) cloning immunoglobulin genes from said hybridoma into a mammalian expression cell; (e) incubating said mammalian expression cell in the presence of at least one chemical inhibitor of mismatch repair, thereby forming a hypermutated mammalian expression cell; (f) performing a screen for hypermutated mammalian expression cells that secrete antibodies with higher affinity for antigen as compared to antibodies produced from said parental mammalian expression cells; and (g) performing a second screen for hypermutated mammalian expression cells that produce higher titers of antibodies than that produced by parental mammalian expression cells; thereby producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0047] In some embodiments of the method of the invention the method may further comprise the removal of the chemical inhibitor from the hypermutated hybridoma and/or hypermutated mammalian expression cells, thereby stabilizing the genome of said hypermutated hybridoma cells and/or hypermutated mammalian expression cells.

[0048] The invention also comprises antibodies produced by the mammalian expression cells.

[0049] In another embodiment, the invention comprises a method for producing hybridoma cells that produce high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*, wherein the donor cells are derived from a donor that is naturally deficient in mismatch repair; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells, wherein the hybridoma cellsare deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; thereby producing hybridoma cells producing high-affinity antibodies.

[0050] The method may further comprise introducing a wild-type gene for mismatch repair into said selected hypermutated hybridoma cell to complement the mismatch repair deficiency, thereby restabilizing the genome of said selected hypermutated hybridoma cell.

[0051] The invention also comprises antibodies produced by the hybridoma cells.

[0052] In another embodiment, the invention comprises a method for producing hybridoma cells that produce high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells, wherein the myeloma cells are naturally deficient in mismatch repair, thereby forming parental hybridoma cells, wherein the hybridoma cells are deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; thereby producing hybridoma cells producing high-affinity antibodies.

[0053] The method may further comprise introducing a wild-type gene for mismatch repair into said selected hypermutated hybridoma cell to complement the mismatch repair deficiency, thereby restabilizing the genome of said selected hypermutated hybridoma cell.

[0054] The invention also comprises antibodies produced by the hybridoma cells.

[0055] In another embodiment, the invention comprises a method for producing hybridoma cells that produce high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells in high titers comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*, wherein the donor cells are derived from a donor that is naturally deficient in mismatch repair; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells, wherein the hybridoma cells are deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; (f) performing a second screen for hypermutated hybridoma cells that produce increased titers of antibodies as compared with parental hybridoma cells; (g) selecting hypermutated hybridoma cells that produce antibodies in higher titers than produced by the

parental hybridoma cells; thereby producing hybridoma cells producing high titers of high-affinity antibodies.

[0056] The method may further comprise introducing a wild-type gene for mismatch repair into said selected hypermutated hybridoma cell to complement the mismatch repair deficiency, thereby restabilizing the genome of said selected hypermutated hybridoma cell.

[0057] The invention also comprises antibodies produced by the hybridoma cells.

[0058] In another embodiment, the invention comprises a method for producing hybridoma cells that produce high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells in high titers comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells, wherein the myeloma cells are naturally deficient in mismatch repair, thereby forming parental hybridoma cells, wherein the hybridoma cells are deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; (f) performing a second screen for hypermutated hybridoma cells that produce increased titers of antibodies as compared with parental hybridoma cells; (g) selecting hypermutated hybridoma cells that produce antibodies in higher titers than produced by the parental hybridoma cells; thereby producing hybridoma cells producing high titers of high-affinity antibodies.

[0059] The method may further comprise introducing a wild-type gene for mismatch repair into said selected hypermutated hybridoma cell to complement the mismatch repair deficiency, thereby restabilizing the genome of said selected hypermutated hybridoma cell.

[0060] The invention also comprises antibodies produced by the hybridoma cells.

[0061] In another embodiment, the invention comprises a method for producing mammalian expression cells that produce high-affinity antibodies in high titers from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*, wherein the donor cells are derived from a donor that is naturally deficient in mismatch repair; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells, wherein the hybridoma cells are deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the

hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; (f) cloning immunoglobulin genes from said hypermutated hybridoma into a mammalian expression cell; thereby producing a mammalian expression cell that produces high titers of high-affinity antibodies in high titer from *in vitro* immunized immunoglobulin-producing cells.

[0062] In some embodiments, the parental mammalian expression cell is further incubated in the presence of at least one chemical inhibitor of mismatch repair, thereby forming a hypermutated mammalian expression cell; and the hypermutated mammalian expression cells are screened for higher production of antibodies than that of the parental mammalian expression cells.

[0063] In some embodiments of the method of the invention the method may further comprise the removal of the chemical inhibitor from the hypermutated mammalian expression cells, thereby stabilizing the genome of said hypermutated mammalian expression cells.

[0064] The invention also comprises antibodies produced by the mammalian expression cells.

[0065] In another embodiment, the invention comprises a method for producing mammalian expression cells that produce high-affinity antibodies in high titer from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells, wherein the myeloma cells are naturally deficient in mismatch repair, thereby forming parental hybridoma cells, wherein the hybridoma cells are deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; and (f) cloning immunoglobulin genes from said hypermutated hybridoma cell into a mammalian expression cell; thereby producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0066] In some embodiments, the parental mammalian expression cell is further incubated in the presence of at least one chemical inhibitor of mismatch repair, thereby forming a hypermutated mammalian expression cell; and the hypermutated mammalian expression cells are screened for higher production of antibodies than that of the parental mammalian expression cells.

[0067] In some embodiments of the method of the invention the method may further comprise the removal of the chemical inhibitor from the hypermutated mammalian expression cells, thereby stabilizing the genome of said hypermutated mammalian expression cells.

[0068] The invention also comprises antibodies produced by the hybridoma cells.

[0069] In some embodiments of the methods of the invention, the immunoglobulin-producing cells are mammalian cells, including but not limited to, mouse cells, rat cells, goat cells, cow cells, horse cells, dog cells, cat cells, rabbit cells, bird cells, monkey cells and human cells. In preferred embodiments, the cells are human cells.

[0070] In some embodiments the dominant negative allele of a mismatch repair gene is a dominant negative allele of *PMS2*, *PMS1*, *PMSR3*, *PMSR2*, *PMSR6*, *MLH1*, *GTBP*, *MSH3*, *MSH2*, *MLH3*, or *MSH1*, and homologs of *PMSR* genes as described in Nicolaides *et al.* (1995) *Genomics* 30:195-206 and Horii *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:1257-1264. However, the mismatch repair genes are not limit to these examples.

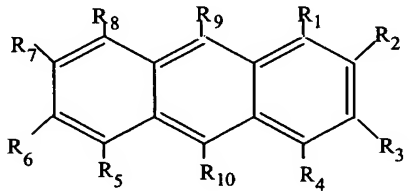
[0071] In some embodiments of the method of the invention, the immunogenic antigen is conjugated to a mitogenic polypeptide comprising at least a portion of a polypeptide including, but not limited to tetanus toxoid, ovalbumin, bovine serum albumen, thyroglobulin, diptheria toxoid, BCG, and cholera toxin. In some embodiments, the antigen is generated by denaturing the mature protein.

[0072] In some embodiments of the method of the invention, the antibodies produced have an affinity of at least about $1 \times 10^7\ M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^8\ M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^9\ M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^{10}\ M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^{11} M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^{12} M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^{13}\ M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^{14}\ M^{-1}$.

[0073] In some embodiments, the antibodies are produced in a higher titer than the parental cell lines, such as in an amount of at least about 1.5 fold higher than the parental cell line. In other embodiments, the titer is at least about 1.5-3 fold higher than the parental cell line. In other embodiments, the titer is at least about 3-5 fold higher than the parental cell line. In other embodiments, the titer is at least about 5-7 fold higher than the parental cell line. In other embodiments, the titer is at least about 7-9 fold higher than the parental cell line. In other embodiments, the titer is at least about 9-10 fold higher than the parental cell line.

[0074] In some embodiments of the method of the invention, mutation rates are further enhanced by incubating the hybridoma cells with a chemical mutagen, such as, but not limited to N-ethyl-N-nitrosourea, N-methyl-N-nitrosourea, procarbazine hydrochloride, chlorambucil, cyclophosphamide, methyl methanesulfonate, ethyl methanesulfonate, diethyl sulfate, acrylamide monomer, triethylene melamin, melphalan, nitrogen mustard, vincristine, dimethylnitrosamine, N-methyl-N'-nitro-nitrosoguanidine, 7,12 dimethylbenz (a) anthracene, ethylene oxide, hexamethylphosphoramide, and bisulfan.

[0075] The chemical inhibitors of mismatch repair used in certain embodiments of the methods of the invention include, but are not limited to, at least one ofan anthracene, an ATPase inhibitor, a nuclease inhibitor, an RNA interference molecule, a polymerase inhibitor and an antisense oligonucleotide that specifically hybridizes to a nucleotide encoding a mismatch repair protein. In some embodiments, the chemical inhibitor is an anthracene having the formula:



wherein R_1-R_{10} are independently hydrogen, hydroxyl, amino, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, O-alkynyl, S-alkynyl, N-alkynyl, aryl, substituted aryl, aryloxy, substituted aryloxy, heteroaryl, substituted heteroaryl, aralkyloxy, arylalkyl, alkylaryl, alkylaryloxy, arylsulfonyl, alkylsulfonyl, alkoxycarbonyl, aryloxycarbonyl, guanidino, carboxy, an alcohol, an amino acid, sulfonate, alkyl sulfonate, CN, NO_2, an aldehyde group, an ester, an ether, a crown ether, a ketone, an organosulfur compound, an organometallic group, a carboxylic acid, an organosilicon or a carbohydrate that optionally contains one or more alkylated hydroxyl groups; wherein said heteroalkyl, heteroaryl, and substituted heteroaryl contain at least one heteroatom that is oxygen, sulfur, a metal atom, phosphorus, silicon or nitrogen; and wherein said substituents of said substituted alkyl, substituted alkenyl, substituted alkynyl, substituted aryl, and substituted heteroaryl are halogen, CN, NO_2, lower alkyl, aryl, heteroaryl, aralkyl, aralkoxy, guanidino, alkoxycarbonyl, alkoxy, hydroxy, carboxy and amino; and wherein said amino groups are optionally substituted with an acyl group, or 1 to 3 aryl or lower alkyl groups. In certain embodiments, R_5 and R_6 are hydrogen. In other embodiments, R_1-R_{10} are independently hydrogen, hydroxyl, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, phenyl,

tolyl, hydroxymethyl, hydroxypropyl, or hydroxybutyl. Non-limiting examples of the anthracenes include 1,2-dimethylanthracene, 9,10-dimethylanthracene, 7,8-dimethylanthracene, 9,10-duphenylanthracene, 9,10-dihydroxymethylanthracene, 9-hydroxymethyl-10-methylanthracene, dimethylanthracene-1,2-diol, 9-hydroxymethyl-10-methylanthracene-1,2-diol, 9-hydroxymethyl-10-methylanthracene-3,4-diol, and 9,10-di-*m*-tolylanthracene.

[0076] The chemical inhibitor may be introduced into the growth medium of the cells. In some embodiments, the chemical inhibitor may be withdrawn from the hypermutated hybridoma cells in order to re-stabilize the genome of the cells.

[0077] The invention also comprises a method for *in vitro* production of antigen-specific immunoglobulin-producing cells comprising: (a) isolating donor cells from an animal; (b) treating said cells with L-leucyl-L-leucine methy ester hydrobromide; (c) incubating said donor cells with an immunogenic antigen *in vitro*, at 25-37°C, 5-10% CO_2, in medium supplemented with 5-15% serum, and a growth promoting cytokine for 4 days; (d) washing said cells in medium; and (e) culturing said cells in medium supplemented with 5-15% serum an additional 8 days; thereby stimulating the production of antigen-specific immunoglobulin-producing cells.

[0078] In some embodiments, the immunoglobulin-producing cells are human cells.

[0079] In some embodiments of the method of the invention, the immunogenic antigen is conjugated to a mitogenic polypeptide comprising at least a portion of a polypeptide including, but not limited to tetanus toxoid, ovalbumin, bovine serum albumen, thyroglobulin, diptheria toxoid, BCG, and cholera toxin. In some embodiments, the antigen is generated by denaturing the mature protein.

[0080] In some embodiments of the method of the invention, the antibodies produced have an affinity of at least about $1 \times 10^7 M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^8 M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^9 M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^{10} M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^{11} M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^{12} M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^{13} M^{-1}$. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^{14} M^{-1}$.

[0081] In some embodiments, the antibodies are produced in a higher titer than the parental cell lines, such as in an amount of at least about 1.5 fold higher than the parental cell line. In other embodiments, the titer is at least about 1.5-3 fold higher than the parental cell line. In

other embodiments, the titer is at least about 3-5 fold higher than the parental cell line. In other embodiments, the titer is at least about 5-7 fold higher than the parental cell line. In other embodiments, the titer is at least about 7-9 fold higher than the parental cell line. In other embodiments, the titer is at least about 9-10 fold higher than the parental cell line.

[0082] In some embodiments of the method of the invention, mutation rates are further enhanced by incubating the hybridoma cells and/or mammalian expression cells with a chemical mutagen, such as, but not limited to N-ethyl-N-nitrosourea, N-methyl-N-nitrosourea, procarbazine hydrochloride, chlorambucil, cyclophosphamide, methyl methanesulfonate, ethyl methanesulfonate, diethyl sulfate, acrylamide monomer, triethylene melamin, melphalan, nitrogen mustard, vincristine, dimethylnitrosamine, N-methyl-N'-nitro-nitrosoguanidine, 7,12 dimethylbenz (a) anthracene, ethylene oxide, hexamethylphosphoramide, and bisulfan.

[0083] The mammalian expression cells used in the methods of the invention may include, but are not limited to, Chinese Hamster Ovary, baby hamster kidney cells, human embryonic kidney line 293, normal dog kidney cell lines, normal cat kidney cell lines, monkey kidney cells, African green monkey kidney cells, COS cells, and non-tumorigenic mouse myoblast G8 cells, fibroblast cell lines, myeloma cell lines, mouse NIH/3T3 cells, $LMTK^{31}$ cells, mouse sertoli cells, human cervical carcinoma cells, buffalo rat liver cells, human lung cells, human liver cells, mouse mammary tumor cells, TRI cells, MRC 5 cells, and FS4 cells.

[0084] These and other embodiments are described more fully in the next section and include certain non-limiting examples.

BRIEF DESCRIPTION OF THE DRAWINGS

[0085] **Figure 1** shows the immune response of PBMCs to antigen stimulation. PBMCs were cultured in the presence or absence of TT for 4 days then washed with medium and cultured in the presence or absence of TT for an additional eight days. Culture supernates were collected and tested for the presence of antibody reactive to TT. Antibodies bound to TT pre-coated on the solid phase were detected with HRP-labeled goat anti-human IgG, or HRP-labeled goat anti-human IgM.

[0086] **Fig. 2A** shows reactivity of donor serum to TT by detection of donor anti-TT IgG. **Fig. 2B** shows reactivity of donor serum to TT by detection of donor anti-TT IgM.

[0087] **Figure 3** shows the frequency of the anti-TT response of PBMCs upon *in vitro* immunization with TT, or with TT in combination with IL-2, or CD40L.

[0088] **Figure 4** shows the intensity of the response of PBMCs upon *in vitro* immunization with TT, or with TT in combination with IL-2, or CD40L.

[0089] **Figure 5** shows the response of hybridomas expressing anti-TT antibodies.

[0090] **Fig. 6A** shows the reactivity of unstimulated PBMCs to EGFR. **Fig. 6B** shows the reactivity of PBMCs to EGFR after immunization with EGFR-TT. **Fig. 6C** shows the reactivity of unstimulated PBMCs to EGFR-TT. **Fig. 6D** shows the reactivity of PBMCs to EGFR-TT after immunization with EGFR-TT.

[0091] **Figure 7** shows the response of hybridomas expressing antibodies against human EGFR. Antibodies bound to EGFR or BSA (control) pre-coated on the solid phase were detected with HRP-labeled goat anti-human IgG or HRP-labeled goat anti-human IgM.

[0092] **Figure 8** shows the IgG and IgM responses of cells immunized with tumor cells *in vitro*.

[0093] **Figure 9** shows reactivity of clones to GM-CSF, chick ovalbumin (CAB), or keyhole limpet hemocyanin.

[0100] **Figure 10** shows inhibitory effect of anti-GM-CSF antibodies on proliferation of TF-1 cells. Shown are the effects of a GM-CSF-specific, blocking antibody; a GM-CSF-specific, non-blocking antibody; and a non-specific antibody.

DETAILED DESCRIPTION OF THE INVENTION

[0101] The referenced patents, patent applications, and scientific literature, including accession numbers to GenBank database sequences, referred to herein are hereby incorporated by reference in their entirety. Any conflict between any reference cited herein and the specific teachings of this specification shall be resolved in favor of the latter. Likewise, any conflict between an art-understood definition of a word or phrase and a definition of the word or phrase as specifically taught in this specification shall be resolved in favor of the latter.

[0102] Standard reference works setting forth the general principles of recombinant DNA technology known to those of skill in the art include, but are not limited to Ausubel *et al.* CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York (1998); Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, 2D ED., Cold Spring Harbor Laboratory Press, Plainview, New York (1989); Kaufman *et al.*, Eds., HANDBOOK OF MOLECULAR AND CELLULAR METHODS IN BIOLOGY AND MEDICINE, CRC Press, Boca Raton (1995); McPherson, Ed., DIRECTED MUTAGENESIS: A PRACTICAL APPROACH, IRL Press, Oxford (1991).

[0103] The invention provides various embodiments of a method for producing antibody-producing cells and antibodies from *in vitro* immunized cells with high affinity, and/or increased production. In some embodiments, the cells that produce the antibodies are

hybridoma cells, formed by fusing myeloma cells with the lymphoid cells that have been immunized against an antigen *in vitro*. In other embodiments, the cells that produce the antibodies are mammalian cells that have been transfected with immunoglobulin genes cloned from lymphoid cells that have been immunized against an antigen *in vitro*. In some embodiments, the method employs both hybridoma cells and mammalian cells. Some basic embodiments of the method of the invention may be described as follows.

[0104] In one embodiment, the invention provides a method for generating hybridoma cells producing high-affinity antibodies from *in vitro* immunized, immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells, wherein the hybridoma cells express a dominant negative allele of a mismatch repair gene; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce antibodies with greater affinity for the antigen than antibodies produced by the parental hybridoma cells; thereby producing hybridoma cells producing high-affinity antibodies.

[0105] In another embodiment, the invention provides methods of producing hybridoma cells that produce high titers of antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells, wherein the hybridoma cells express a dominant negative allele of a mismatch repair gene; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen of the hypermutated hybridoma cells for antigen-specific antibodies produced in higher titers than that produced by the parental hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce higher titers of antibodies than that produced by the parental hybridoma cells; thereby producing hybridoma cells that produce high titers of antibodies.

[0106] In another embodiment, the invention provides a method for producing hybridoma cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing said immunoglobulin-producing cells with myeloma cells to form hybridoma cells; (c) performing

a screen for binding of antibodies produced from said hybridoma cells to antigen; (d) cloning immunoglobulin genes from said hybridoma into a mammalian expression cell, wherein said mammalian expression cell expresses a dominant negative allele of a mismatch repair gene; and (e) performing a screen for mammalian expression cells that secrete antibodies with higher affinity for antigen as compared to antibodies produced from said hybridoma cells; thereby producing hybridoma cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0107] In another embodiment, the invention provides a method for producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells are produced by: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing said immunoglobulin-producing cells with myeloma cells to form hybridoma cells, wherein said hybridoma cells express a dominant negative allele of a mismatch repair gene; (c) incubating said parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from said hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with greater affinity for said antigen than antibodies produced by said parental hybridoma cells; and (f) cloning immunoglobulin genes from said hybridoma into a mammalian expression cell; thereby producing high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0108] In yet another embodiment, the invention provides mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells are produced by: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form hybridoma cells; (c) performing a screen for binding of antibodies produced from the hybridoma cells to antigen; (d) cloning immunoglobulin genes from the hybridoma into a parental mammalian expression cell, wherein the mammalian expression cell expresses a dominant negative allele of a mismatch repair gene; (e) incubating the parental mammalian expression cell to allow for mutagenesis, thereby forming hypermutated mammalian expression cells; (f) performing a screen of hypermutable mammalian expression cells that secrete antibodies with higher affinity for antigen as compared to antibodies produced from the hybridoma cells; and (g) performing a screen of hypermutable mammalian expression cells that secrete higher titers of antibodies than parental mammalian expression cells; thereby producing mammalian expression cells that

produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0109] In yet another embodiment, the invention provides a method of producing hybridoma cells that produce high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells are produced by: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells; (c) incubating the parental hybridoma cells in the presence of at least one chemical inhibitor of mismatch repair, thereby forming hypermutated hybridoma cells; (d) performing a screen for antigen binding for antibodies produced from the hypermutated hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce antibodies with greater affinity for the antigen than antibodies produced by said parental hybridoma cells; thereby producing hybridoma cells that produce high-affinity antibodies.

[0110] In still another embodiment, the invention provides a method of producing hybridoma cells that produce high titers of antibodies from *in vitro* immunized immunoglobulin-producing cells are produced by: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells; (c) incubating the parental hybridoma cells in the presence of at least one chemical inhibitor of mismatch repair, thereby forming hypermutated hybridoma cells; (d) performing a screen of the hypermutated hybridoma cells for antigen-specific antibodies produced in higher titers than that produced by the parental hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce higher titers of antibodies than that produced by said parental hybridoma cells; thereby producing hybridoma cells producing high titers of antibodies.

[0111] In another embodiment, the invention provides methods for producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells are produced by: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form hybridoma cells; (c) performing a screen for antigen binding of antibodies produced from the hybridoma cells; (d) cloning immunoglobulin genes from the hybridoma cells into a mammalian expression cell; (e) incubating the mammalian expression cell in the presence of at least one chemical inhibitor of mismatch repair; and (f) performing a screen for mammalian expression cells that secrete antibodies with higher affinity for antigen as compared to antibodies produced from the

hybridoma cells; thereby producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0112] In yet another embodiment, the invention provides a method for producing mammalian expression cells that produce high affinity antibodies to a selected antigen from *in vitro* immunized immunoglobulin-producing cells are produced in high titers by: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells to form hybridoma cells; (c) incubating the hybridoma cells in the presence of at least one chemical inhibitor of mismatch repair to form hypermutated hybridoma cells; (d) performing a screen for antigen binding for antibodies produced from the hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with greater affinity for the antigen than antibodies produced by the parental hybridoma cells; and (f) cloning immunoglobulin genes from the hypermutated hybridoma cells into a mammalian expression cell, thereby forming parental mammalian expression cells; thereby producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0113] In yet another embodiment, the invention also provides methods for producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing said immunoglobulin-producing cells with myeloma cells to form hybridoma cells; (c) performing a screen for binding of antibodies produced from said hybridoma cells to antigen; (d) cloning immunoglobulin genes from said hybridoma into a mammalian expression cell; (e) incubating said mammalian expression cell in the presence of at least one chemical inhibitor of mismatch repair, thereby forming a hypermutated mammalian expression cell; (f) performing a screen for hypermutated mammalian expression cells that secrete antibodies with higher affinity for antigen as compared to antibodies produced from said parental mammalian expression cells; and (g) performing a second screen for hypermutated mammalian expression cells that produce higher titers of antibodies that produced by parental mammalian expression cells; thereby producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0114] In another embodiment, the invention comprises a method for producing hybridoma cells that produce high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing

cells with an immunogenic antigen *in vitro*, wherein the donor cells are derived from a donor that is naturally deficient in mismatch repair; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells, wherein the hybridoma cellsare deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; thereby producing hybridoma cells producing high-affinity antibodies.

[0115] In another embodiment, the invention comprises a method for producing hybridoma cells that produce high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells, wherein the myeloma cells are naturally deficient in mismatch repair, thereby forming parental hybridoma cells, wherein the hybridoma cells are deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; and (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; thereby producing hybridoma cells producing high-affinity antibodies.

[0116] In another embodiment, the invention comprises a method for producing hybridoma cells that produce high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells in high titers comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*, wherein the donor cells are derived from a donor that is naturally deficient in mismatch repair; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells, wherein the hybridoma cells are deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; (f) performing a second screen for hypermutated hybridoma cells that produce increased titers of antibodies as compared with parental hybridoma cells; and (g)

selecting hypermutated hybridoma cells that produce antibodies in higher titers than produced by the parental hybridoma cells; thereby producing hybridoma cells producing high titers of high-affinity antibodies.

[0117] In another embodiment, the invention comprises a method for producing hybridoma cells that produce high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells in high titers comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells, wherein the myeloma cells are naturally deficient in mismatch repair, thereby forming parental hybridoma cells, wherein the hybridoma cells are deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; (f) performing a second screen for hypermutated hybridoma cells that produce increased titers of antibodies as compared with parental hybridoma cells; and (g) selecting hypermutated hybridoma cells that produce antibodies in higher titers than produced by the parental hybridoma cells; thereby producing hybridoma cells producing high titers of high-affinity antibodies.

[0118] In another embodiment, the invention comprises a method for producing mammalian expression cells that produce high-affinity antibodies in high titers from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*, wherein the donor cells are derived from a donor that is naturally deficient in mismatch repair; (b) fusing the immunoglobulin-producing cells with myeloma cells to form parental hybridoma cells, wherein the hybridoma cells are deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; and (f) cloning immunoglobulin genes from said hypermutated hybridoma into a mammalian expression cell; thereby producing a mammalian expression cell that produce high titers of high-affinity antibodies in high titer from *in vitro* immunized immunoglobulin-producing cells.

[0119] In another embodiment, the invention comprises a method for producing mammalian expression cells that produce high-affinity antibodies in high titer from *in vitro* immunized immunoglobulin-producing cells comprising: (a) combining donor cells comprising immunoglobulin-producing cells with an immunogenic antigen *in vitro*; (b) fusing the immunoglobulin-producing cells with myeloma cells, wherein the myeloma cells are naturally deficient in mismatch repair, thereby forming parental hybridoma cells, wherein the hybridoma cells are deficient in mismatch repair; (c) incubating the parental hybridoma cells to allow for mutagenesis, thereby forming hypermutated hybridoma cells; (d) performing a screen for binding of antibodies to antigen for antibodies produced from the hypermutated hybridoma cells; (e) selecting hypermutated hybridoma cells that produce antibodies with enhanced affinity for the antigen than antibodies produced by the parental hybridoma cells; and (f) cloning immunoglobulin genes from said hypermutated hybridoma cell into a mammalian expression cell; thereby producing mammalian expression cells that produce high titers of high-affinity antibodies from *in vitro* immunized immunoglobulin-producing cells.

[0120] The invention also provides hybridoma cells, expression cells produced by any of the methods of the invention, as well as antibodies produced by any of the hybridoma cells and expression cells of the invention.

[0121] In still another embodiment, antigen-specific immunoglobulin-producing cells are produced by: (a) isolating donor cells from an animal; (b) treating said cells with L-leucyl-L-leucine methy ester hydrobromide; (c) incubating said donor cells with an immunogenic antigen *in vitro*, at 25-37°C, 5-10% CO_2, in medium supplemented with 5-15% serum, and a growth promoting cytokine for 4 days; (d) washing said cells in medium; and (e) culturing said cells in medium supplemented with 5-15% serum an additional 8 days; thereby stimulating the production of antigen-specific immunoglobulin-producing cells.

[0122] The blood cells used in the methods of the invention may be derived from any animal that produces antibodies. Preferably, the donor cells are derived from mammals, including, but not limited to humans, monkeys, mice, rats, guinea pigs, hamsters, gerbils, birds, rabbits, sheep, goats, pigs, horses, and cows. The source of blood is not necessarily limited, but may be whole blood or fractions containing lymphocytes. The blood may be donor or cord blood, for example. In some embodiments, the blood cells are preferably human donor cells.

[0123] The myeloma cells used to create the hybridoma cells in the method of the invention may be derived from any species known to have suitable myeloma cells. For example, but not by way of limitation, the myeloma cells may be conveniently derived from humans or mice. Suitable examples of myeloma cells include, but are not limited to the HuNS1 myeloma as

described in U.S. Patent No. 4,720,459 to Winkelhake, and deposited with the American Type Culture Collection (ATCC) as CRL 8644; GM4672; RPMI 8226; and murine myeloma cell lines (*e.g.*, P3-NS1/1-Ag4-1; P3-x63-Ag8.653; Sp2/O-Ag14; NS/O, NS/1, SP2 and S194).

[0124] The mammalian expression cells suitable for use in certain embodiments of the method of the invention include, but are not limited to Chinese Hamster Ovary cells (CHO cells, Urlaub and Chasin (1980) *Proc. Natl. Acad. Sci. USA*, 77: 4216), baby hamsterkidney (BHK cells), human embryonic kidney line 293 (HeLa cells, Graham *et al.*, (1977) *J. Gen Virol.*, 36: 59), normal dog kidney cell line (*e.g.*, MDCK, ATCC CCL 34), normal cat kidney cell line (CRFK cells), monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587), COS (*e.g.*, COS-7) cells, and non-tumorigenic mouse myoblast G8 cells (*e.g.*, ATCC CRL 1246), fibroblast cell lines (*e.g.*, human, murine or chicken embryo fibroblast cell lines), myeloma cell lines, mouse NIH/3T3 cells, $LMTK^{31}$ cells, mouse sertoli cells (TM4, Mather, (1980) *Biol. Reprod.*, 23:243-251); human cervical carcinoma cells (HELA, ATCC CCL 2); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor cells (MMT 060562, ATCC CCL51), TRI cells (Mather *et al* (1982) *Annals N.Y. Acad. Sci.* 383:44-68); MRC 5 cells; FS4 cells; and the human hepatoma line (Hep G2).

[0125] As an alternative to mammalian expression cells, other non-mammalian cells may be used to express the cloned immunoglobulin genes. Such non-mammalian cells include, but are not limited to insect cells (*e.g.*, *Spodoptera frugiperda* cells and the like). Vectors and non-mammalian host cells are well known in the art and are continually being optimized and developed. Any host cell system capable of expressing antibodies may be used in the methods of the invention.

[0126] As used herein, "dominant negative allele of a mismatch repair gene" refers to an allele of a mismatch repair gene that, when expressed, exerts a dominant phenotype in the cell or organism that leads to an inhibition of the mismatch repair system, even in the presence of a wild-type allele. Cells expressing a dominant negative allele of a mismatch repair gene are hypermutable and accumulate mutations at a higher rate than wild-type cells. Examples of nucleic acid sequences encoding mismatch repair proteins useful in the method of the invention include, but are not limited to the following: *PMS1* (SEQ ID NO:1); *PMS2* (SEQ ID NO:3); *PMS2-134* (SEQ ID NO:5); *PMSR2* (SEQ ID NO:7); *PMSR3* (SEQ ID NO:9); *MLH1* (SEQ ID NO:11); *MLH3* (SEQ ID NO:13); *MSH2* (SEQ ID NO:15); *MSH3* (SEQ ID NO:17); *MSH4* (SEQ ID NO:19); *MSH5* (SEQ ID NO:21); *MSH6* (SEQ ID NO:23); *PMSR6* (SEQ ID NO:25); *PMSL9* (SEQ ID NO:27); yeast *MLH1* (SEQ ID NO:29); mouse *PMS2* (SEQ ID

NO:31); mouse *PMS2-134* (SEQ ID NO:33); *Arabidopsis thaliana PMS2* (SEQ ID NO:35); *A. thaliana PMS2-134* (SEQ ID NO:37) *A. thaliana PMS1* (SEQ ID NO:39); *A. thaliana MSH7* (SEQ ID NO:41) *A. thaliana MSH2* (SEQ ID NO:43); *A. thaliana MSH3* (SEQ ID NO:45); *A. thaliana MSH6-1* (SEQ ID NO:47); and *Oryza satvia MLH1* (SEQ ID NO:49). The corresponding amino acid sequences for the listed nucleic acid sequences are: PMS1 (SEQ ID NO:2); PMS2 (SEQ ID NO:4); PMS2-134 (SEQ ID NO:6); PMSR2 (SEQ ID NO:8); PMSR3 (SEQ ID NO:10); MLH1 (SEQ ID NO:12); MLH3 (SEQ ID NO:14); MSH2 (SEQ ID NO:16); MSH3 (SEQ ID NO:18); MSH4 (SEQ ID NO:20); MSH5 (SEQ ID NO:22); MSH6 (SEQ ID NO:24); PMSR6 (SEQ ID NO:26); PMSL9 (SEQ ID NO:28); yeast MLH1 (SEQ ID NO:30); mouse PMS2 (SEQ ID NO:32); mouse PMS2-134 (SEQ ID NO:34); *Arabidopsis thaliana* PMS2 (SEQ ID NO:36); *A. thaliana* PMS2-134 (SEQ ID NO:38); *A. thaliana* PMS1 (SEQ ID NO:40); *A. thaliana* MSH7 (SEQ ID NO:42) *A. thaliana* MSH2 (SEQ ID NO:44); *A. thaliana* MSH3 (SEQ ID NO:46); *A. thaliana* MSH6-1 (SEQ ID NO:48); and *Oryza satvia* MLH1 (SEQ ID NO:50).

[0127] As used herein, "high titer" refers to an titer of at least about 1.5 fold higher than the parental cell line. In some embodiments, the titer is at least about 1.5-3 fold higher, 3-5 fold higher, 5-7 fold higher, 7-9 fold higher, or 9-10 fold higher than the parental cell line.

[0128] As used herein, "high affinity" refers to a high antibody binding affinity, that may be calculated according to standard methods by the formula $K_a = 8/3$ (It-Tt) where "It" is the total molar concentration of inhibitor uptake at 50% tracer and "Tt" is the total molar concentration of tracer. See Muller (1980) *J. Immunol. Meth.* 34:345-352. Binding affinity may also be calculated using the formula $B/T = n \cdot N_{Ab} \cdot W^{108} [(V-V_m)K+Q \cdot W]$ (See Antoni and Mariani (1985) *J. Immunol. Meth.* 83:61-68). As used herein, "high affinity" is at least about 1×10^7 M^{-1}. In some embodiments, the antibodies have an affinity of at least about 1×10^8 M^{-1}. In other embodiments, the antibodies have an affinity of at least about $1 \times 10^9 M^{-1}$. In other embodiments, the antibodies have an affinity of at least about 1×10^{10} M^{-1}. In other embodiments, the antibodies have an affinity of at least about 1×10^{11} M^{-1}. In other embodiments, the antibodies have an affinity of at least about 1×10^{12} M^{-1}. In other embodiments, the antibodies have an affinity of at least about 1×10^{13} M^{-1}. In other embodiments, the antibodies have an affinity of at least about 1×10^{14} M^{-1}.

[0129] As used herein, "antigen-specific" refers to an interaction between the CDR regions of the immunoglobulin molecule with an epitope of the antigen wherein the CDR regions of the immunoglobulin molecule binds to the epitope.

[0130] As used herein, "cured" refers to a state of the cells wherein the dominant negative mismatch repair gene has been eliminated from the cell or wherein the expression of the dominant negative allele has been turned off, leading to a stabilized genome, producing stable biological products such as immunoglobulins.

[0131] In some embodiments of the methods of the invention, mismatch repair is inhibited by introducing a dominant negative allele of a mismatch repair gene into a cell.

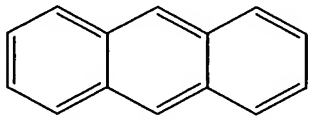
[0132] In other embodiments of the methods of the invention, mismatch repair is inhibited by exposing cells that express an antibody to a compound that inhibits mismatch repair. In some embodiments, the compound is an ATPase inhibitor. Suitable ATPase inhibitors include, but not limited to ATP analogs that are capable of blocking the ATPase activity necessary for mismatch repair in the cell. Examples of ATP analogs that may be used in the methods of the invention, include, but are not limited to non-hydrolyzable forms of ATP, such as AMP-PNP and ATPγS, which block mismatch repair activity (Galio *et al.* (1999) *Nucl. Acids Res.* 27:2325-2331; Allen *et al.* (1997) *EMBO J.* 16:4467-4476; Bjornson *et al.* (2000) *Biochem.* 39:3176-3183). Other suitable ATPase inhibitors may be identified using mismatch repair reporter cells that may be screened with candidate ATPase inhibitors to identify those compounds which effectively block ATPase activity in the cells.

[0133] In other embodiments of the methods of the invention, mismatch repair is inhibited by exposing cells that express an antibody to a nuclease inhibitor. The nuclease inhibitors are capable of blocking exonuclease activity in the mismatch repair biochemical pathway. Mismatch repair reporter cells may be screened with candidate nuclease inhibitors to identify compounds that effectively block the exonuclease activity of the mismatch repair system. Suitable nuclease inhibitors which may be used in the methods of the invention include, but are not limited to analogs of N-ethylmaleimide, an endonuclease inhibitor (Huang *et al.* (1995) *Arch. Biochem. Biophys.* 316:485); heterodimeric adenosine-chain-acridine compounds, exonuclease III inhibitors (Belmont *et al.* (2000) *Bioorg. Med. Chem Lett.* 10:293-295); as well as antibiotic compounds such as heliquinomycin, which have helicase inhibitory activity (Chino *et al.* (1998) *J. Antibiot. (Tokyo)* 51:480-486). Other suitable nuclease inhibitors may be identified using mismatch repair reporter cells that may be screened with candidate nuclease inhibitors to identify those compounds which effectively block nuclease activity in the cells.

[0134] In other embodiments of the methods of the invention, mismatch repair is inhibited by exposing the cells producing antibodies to DNA polymerase inhibitors. DNA polymerase inhibitors are capable of blocking the polymerization of DNA which is required for functional

mismatch repair. Examples of suitable DNA polymerase inhibitors include, but are not limited to actinomycin D (Martin *et al.* (1990) *J. Immunol.* 145:1859); aphidicolin (Kuwakado *et al.* (1993) *Biochem. Pharmacol.* 46:1909); 1-(2'-deoxy-2'-fluoro-beta-L-arabinofuranosyl)-5-methyluracil (L-FMAU) (Kukhanova *et al.* (1998) *Biochem. Pharmacol.* 55:1181-1187); and 2'3'-dideoxyribonucleoside 5'-triphosphates (ddNTPs) (Ono *et al.* (1984) *Biomed. Pharmacother.* 38:382-389). Other suitable DNA polymerase inhibitors may be identified using mismatch repair reporter cells that may be screened with candidate DNA polymerase inhibitors to identify those compounds which effectively block DNA polymerase activity in the cells.

[0135] In other embodiments of the methods of the invention, mismatch repair is inhibited by exposing the cells producing antibody to an anthracene. As used herein the term "anthracene" refers to the compound anthracene. However, when referred to in the general sense, such as "anthracenes," "an anthracene" or "the anthracene," such terms denote any compound that contains the fused triphenyl core structure of anthracene, i.e.,



regardless of extent of substitution. The anthracene may be substituted or unsubstituted.

[0136] As used herein, "alkyl" refers to a hydrocarbon containing from 1 to about 20 carbon atoms. Alkyl groups may straight, branched, cyclic, or combinations thereof. Alkyl groups thus include, by way of illustration only, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, cyclopentyl, cyclopentylmethyl, cyclohexyl, cyclohexylmethyl, and the like. Also included within the definition of "alkyl" are fused and/or polycyclic aliphatic cyclic ring systems such as, for example, adamantane. As used herein the term "alkenyl" denotes an alkyl group having at least one carbon-carbon double bond. As used herein the term "alkynyl" denotes an alkyl group having at least one carbon-carbon triple bond.

[0137] In some preferred embodiments, the alkyl, alkenyl, alkynyl, aryl, aryloxy, and heteroaryl substituent groups described above may bear one or more further substituent groups; that is, they may be "substituted". In some preferred embodiments these substituent groups can include halogens (for example fluorine, chlorine, bromine and iodine), CN, NO_2, lower alkyl groups, aryl groups, heteroaryl groups, aralkyl groups, aralkyloxy groups, guanidino, alkoxycarbonyl, alkoxy, hydroxy, carboxy and amino groups. In addition, the alkyl and aryl portions of aralkyloxy, arylalkyl, arylsulfonyl, alkylsulfonyl, alkoxycarbonyl, and aryloxycarbonyl groups also can bear such substituent groups. Thus, by way of example only,

substituted alkyl groups include, for example, alkyl groups fluoro-, chloro-, bromo- and iodoalkyl groups, aminoalkyl groups, and hydroxyalkyl groups, such as hydroxymethyl, hydroxyethyl, hydroxypropyl, hydroxybutyl, and the like. In some preferred embodiments such hydroxyalkyl groups contain from 1 to about 20 carbons.

[0138] As used herein the term "aryl" means a group having 5 to about 20 carbon atoms and which contains at least one aromatic ring, such as phenyl, biphenyl and naphthyl. Preferred aryl groups include unsubstituted or substituted phenyl and naphthyl groups. The term "aryloxy" denotes an aryl group that is bound through an oxygen atom, for example a phenoxy group.

[0139] In general, the prefix "hetero" denotes the presence of at least one hetero (i.e., non-carbon) atom, which is in some preferred embodiments independently one to three O, N, S, P, Si or metal atoms. Thus, the term "heteroaryl" denotes an aryl group in which one or more ring carbon atom is replaced by such a heteroatom. Preferred heteroaryl groups include pyridyl, pyrimidyl, pyrrolyl, furyl, thienyl, and imidazolyl groups.

[0140] The term "aralkyl" (or "arylalkyl") is intended to denote a group having from 6 to 15 carbons, consisting of an alkyl group that bears an aryl group. Examples of aralkyl groups include benzyl, phenethyl, benzhydryl and naphthylmethyl groups.

[0141] The term "alkylaryl" (or "alkaryl") is intended to denote a group having from 6 to 15 carbons, consisting of an aryl group that bears an alkyl group. Examples of aralkyl groups include methylphenyl, ethylphenyl and methylnaphthyl groups.

[0142] The term "arylsulfonyl" denotes an aryl group attached through a sulfonyl group, for example phenylsulfonyl. The term "alkylsulfonyl" denotes an alkyl group attached through a sulfonyl group, for example methylsulfonyl.

[0143] The term "alkoxycarbonyl" denotes a group of formula -C(=O)-O-R where R is alkyl, alkenyl, or alkynyl, where the alkyl, alkenyl, or alkynyl portions thereof can be optionally substituted as described herein.

[0144] The term "aryloxycarbonyl" denotes a group of formula -C(=O)-O-R where R is aryl, where the aryl portion thereof can be optionally substituted as described herein.

[0145] The terms "arylalkyloxy" or "aralkyloxy" are equivalent, and denote a group of formula -O-$R^{/}$-$R^{//}$, where $R^{/}$ is R is alkyl, alkenyl, or alkynyl which can be optionally substituted as described herein, and wherein $R^{//}$ denotes a aryl or substituted aryl group.

[0146] The terms "alkylaryloxy" or "alkaryloxy" are equivalent, and denote a group of formula -O-$R^{/}$-$R^{//}$, where $R^{/}$ is an aryl or substituted aryl group, and $R^{//}$ is alkyl, alkenyl, or alkynyl which can be optionally substituted as described herein.

[0147] As used herein, the term "aldehyde group" denotes a group that bears a moiety of formula -C(=O)-H. The term "ketone" denotes a moiety containing a group of formula -R-C(=O)-R=, where R and R= are independently alkyl, alkenyl, alkynyl, aryl, heteroaryl, aralkyl, or alkaryl, each of which may be substituted as described herein.

[0148] As used herein, the term "ester" denotes a moiety having a group of formula-R-C(=O)-O-R= or -R-O-C(=O)-R= where R and R= are independently alkyl, alkenyl, alkynyl, aryl, heteroaryl, aralkyl, or alkaryl, each of which may be substituted as described herein.

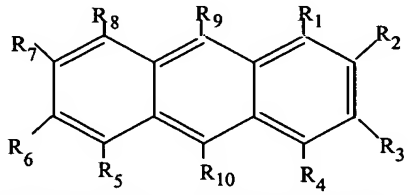
[0149] The term "ether" denotes a moiety having a group of formula -R-O-R= or where R and R= are independently alkyl, alkenyl, alkynyl, aryl, heteroaryl, aralkyl, or alkaryl, each of which may be substituted as described herein.

[0150] The term "crown ether" has its usual meaning of a cyclic ether containing several oxygen atoms. As used herein the term "organosulfur compound" denotes aliphatic or aromatic sulfur containing compounds, for example thiols and disulfides. The term "organometallic group" denotes an organic molecule containing at least one metal atom.

[0151] The term "organosilicon compound" denotes aliphatic or aromatic silicon containing compounds, for example alkyl and aryl silanes.

[0152] The term "carboxylic acid" denotes a moiety having a carboxyl group, other than an amino acid.

[0153] Suitable anthracenes that may be used in the method of the invention comprise compounds having the formula:



wherein R_1-R_{10} are independently hydrogen, hydroxyl, amino, alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, O-alkynyl, S-alkynyl, N-alkynyl, aryl, substituted aryl, aryloxy, substituted aryloxy, heteroaryl, substituted heteroaryl, aralkyloxy, arylalkyl, alkylaryl, alkylaryloxy, arylsulfonyl, alkylsulfonyl, alkoxycarbonyl, aryloxycarbonyl, guanidino, carboxy, an alcohol, an amino acid, sulfonate, alkyl sulfonate, CN, NO_2, an aldehyde group, an ester, an ether, a crown ether, a ketone, an organosulfur compound, an organometallic group, a carboxylic acid, an organosilicon or a carbohydrate that optionally contains one or more alkylated hydroxyl groups; wherein said heteroalkyl, heteroaryl, and substituted heteroaryl contain at least one heteroatom that is oxygen, sulfur, a metal atom, phosphorus, silicon or nitrogen; and wherein

said substituents of said substituted alkyl, substituted alkenyl, substituted alkynyl, substituted aryl, and substituted heteroaryl are halogen, CN, NO_2, lower alkyl, aryl, heteroaryl, aralkyl, aralkoxy, guanidino, alkoxycarbonyl, alkoxy, hydroxy, carboxy and amino; and wherein said amino groups are optionally substituted with an acyl group, or 1 to 3 aryl or loweralkyl groups. In some embodiments, the R_5 and R_6 are hydrogen. In other embodiments, R_1-R_{10} are independently hydrogen, hydroxyl, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, phenyl, tolyl, hydroxymethyl, hydroxypropyl, or hydroxybutyl. Suitable anthracenes for use in the methods of the invention include, but are not limited to 1,2-dimethylanthracene, 9,10-dimethylanthracene, 7,8-dimethylanthracene, 9,10-duphenylanthracene, 9,10-dihydroxymethylanthracene, 9-hydroxymethyl-10-methylanthracene, dimethylanthracene-1,2-diol, 9-hydroxymethyl-10-methylanthracene-1,2-diol, 9-hydroxymethyl-10-methylanthracene-3,4-diol, and 9,10-di-*m*-tolylanthracene.

[0154] Other suitable anthracenes may be identified using mismatch repair reporter cells that may be screened with candidate anthracenes to identify those compounds which effectively block mismatch repair activity in the cells. In some embodiments, the chemical inhibitor of mismatch repair is an RNA interference molecule that is homologous to a mismatch repair gene of the invention. The technique for generating sequence-specific RNA interference molecules is well-known in the art and may be found in, for example, Sharp *et al.* (2000) *Science* 287:2431-2433; Marx (2000) *Science* 288:1370-1372; Grishok *et al.* (2001) *Science* 287:2494-2497; and Fire *et al.* (1998) *Nature* 391:806-811, the disclosures of which are specifically incorporated by reference in their entirety.

[0155] In other embodiments of the method of the invention, mismatch repair is inhibited by exposing the cells producing antibody to "antisense compounds" which specifically hybridize with one or more nucleic acids encoding a mismatch repair gene. As used herein, the terms "target nucleic acid" and "nucleic acid encoding a mismatch repair gene" encompass DNA encoding a mismatch repair gene, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an antisense compound with its target nucleic acid interferes with the normal function of the nucleic acid, such as replication and transcription. The functions of RNA disrupted by antisense compounds include such functions as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, and splicing of the RNA to yield one or more mRNA species. The antisense compound thereby inhibits the expression or function of a mismatch repair gene.

[0156] It is preferred to target specific nucleic acids for antisense inhibition of mismatch repair in order to reversibly disrupt the function of a given mismatch repair gene. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process, beginning with the identification of a nucleic acid sequence whose function is to be modulated. As disclosed herein, there are several mismatch repair genes that may be targeted by an antisense strategy. Among the various mismatch repair genes that may be targeted are *PMS2*, *PMS1*, *PMSR3*, *PMSR2*, *PMSR6*, *MLH1*, *GTBP*, *MSH3*, *MSH2*, *MLH3*, or *MSH1*, and homologs of *PMSR* genes as described in Nicolaides *et al.* (1995) *Genomics* 30:195-206 and Horii *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:1257-1264, including DNA or RNA. The next step of targeting involves the determination of a site or sites within this gene for the antisense interaction to occur, such that inhibition of the function of the mismatch repair gene occurs. In one embodiment, an intragenic site is targeted. An "intragenic site" is a region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine in eukaryotes. It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "startcodon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding a mismatch repair gene, regardless of the sequence(s) of such codons.

[0157] It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, *i.e.*, 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (*i.e.*, 5' or 3') from a translation initiation codon. Similarly, the terms "stop codonregion" and "translation termination codon region" refer to a portion of such an mRNA or gene that

encompasses from about 25 to about 50 contiguous nucleotides in either direction (*i.e.*, 5' or 3') from a translation termination codon.

[0158] The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

[0159] Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites,*i.e.*, intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

[0160] Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, *i.e.*, hybridize sufficiently well and with sufficient specificity, to give the desired effect.

[0161] In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two

nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed. Complementarity of the antisense oligonucleotide is preferably 100%, however, degeneracy may be introduced into the oligonucleotide such that the complementarity, in some embodiments, is 80-85%, 85-90%, 90-95% or 95-100%.

[0162] Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of these compounds are herein below identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary comprise the region of PMS2, for example, which inhibits the translation of the C-terminal portion of the PMS2 protein, effectively forming a truncation mutant. The region targeted comprises a portion of the PMS2 gene that encodes the 134 amino acid of PMS2, for example.

[0163] In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example,

enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

[0164] While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e., from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression. In some embodiments, the oligonucleotides are at least about 15 nucleotides in length and may be at least 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more nucleotides in length.

[0165] In some embodiments, the antisense oligonucleotides comprise a sequence that is complementary to a portion of the mismatch repair sequence shown in SEQ ID NO:1; SEQ ID NO:3; SEQ ID NO:5; SEQ ID NO:7; SEQ ID NO:9; SEQ ID NO:11; SEQ ID NO:13; SEQ ID NO:15; SEQ ID NO:17; SEQ ID NO:19; SEQ ID NO:21; SEQ ID NO:23; SEQ ID NO:25; SEQ ID NO:27; SEQ ID NO:29; SEQ ID NO:31; SEQ ID NO:33; SEQ ID NO:35; SEQ ID NO:37; SEQ ID NO:39; SEQ ID NO:41; SEQ ID NO:43; SEQ ID NO:45; SEQ ID NO:47; or SEQ ID NO:49. In certain embodiments, the oligonucleotide is at least 15-50 nucleotides in length with 85-100% complementarity.

[0166] As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

[0167] Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

[0168] Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage, *i.e.*, a single inverted nucleoside residue which may be a basic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

[0169] Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, each of which is herein incorporated by reference.

[0170] Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino

backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

[0171] Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, each of which is herein incorporated by reference.

[0172] In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et al.*, (1991) *Science* 254:1497-1500.

[0173] Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH_2 --NH--O--CH_2--, --CH_2--N(CH_3)--O--CH_2-- [known as a methylene (methylimino) or MMI backbone], --CH_2--O--N(CH_3)--CH_2 --, --CH_2--N(CH_3)--N(CH_3)--CH_2 -- and --O--N(CH_3)--CH_2 --CH_2-- [wherein the native phosphodiester backbone is represented as --O--P--O--CH_2 --] of the above referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above referenced U.S. Pat. No. 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

[0174] Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are O[$(CH_2)_n$ O]$_m$$CH_3$, O($CH_2$)$_nOCH_3$, O($CH_2$)$_n$$NH_2$,

$O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2 CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O--$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.* (1995) *Helv. Chim. Acta* 78:486-504) *i.e.*, an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, *i.e.*, a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-O--CH_2 --O--CH_2--$N(CH_2)_2$, also described in examples hereinbelow.

[0175] A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methelyne (--CH_2--)$_n$ group bridging the 2' oxygen atom and the 3' or 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

[0176] Other preferred modifications include 2'-methoxy (2'-O--CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2--CH=CH_2), 2'-O-allyl (2'-O--CH_2--CH=CH_2) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, each of which is herein incorporated by reference in its entirety.

[0177] Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (--C≡C--CH_2) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g., 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in THECONCISE ENCYCLOPEDIA OF POLYMER SCIENCE AND ENGINEERING, Kroschwitz, (Ed.) John Wiley & Sons, 1990, pages 858-859, those disclosed by Englisch *et al.* (1991) *Angewandte Chemie* (International Edition) 30:613, and those disclosed by Sanghvi, ANTISENSE RESEARCH AND APPLICATIONS, Crooke and Lebleu (Eds.), CRC Press, 1993, pages 289-302. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C. (Sanghvi, ANTISENSE RESEARCH AND APPLICATIONS, Crooke and Lebleu (Eds.), CRC Press, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

[0178] Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos. 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,750,692; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, each of which is herein incorporated by reference in its entirety.

[0179] Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds ofthe invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed Oct. 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger*et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6553-6556), cholic acid (Manoharan *et al.* (1994) *Bioorg. Med. Chem. Let.* 4:1053-1060), a thioether, *e.g.*, hexyl-S-tritylthiol (Manoharan *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:306-309; Manoharan *et al.* (1993) *Bioorg. Med. Chem. Let.* 3:2765-2770), a thiocholesterol (Oberhauser *et al.* (1992) *Nucl. Acids Res.* 20:533-538), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al.* (1991) EMBO J. 10:1111-1118; Kabanov *et al.* (1990) *FEBS Lett.* 259:327-330; Svinarchuk *et al.* (1993) *Biochimie* 75:49-54), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al.* (1995) *Tetrahedron Lett.* 36:3651-3654; Shea *et al.* (1990) *Nucl. Acids Res.* 18:3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al.* (1995) *Nucleosides & Nucleotides* 14:969-973), or adamantane acetic

acid (Manoharan *et al.* (1995) *Tetrahedron Lett.* 36:3651-3654), a palmityl moiety (Mishra *et al.* (1995) *Biochim. Biophys. Acta* 1264:229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.* (1996) *J. Pharmacol. Exp. Ther.* 277:923-937.

[0180] Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873;5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, each of which is herein incorporated by reference in its entirety.

[0181] It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

[0182] Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or

oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Pat. Nos. 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, each of which is herein incorporated by reference in its entirety.

[0183] As used herein "donor cells comprising immunoglobulin-producing cells" or "donor cells comprising immunoglobulin-producing cells" sometimes referred to simply as "donor cells" or "donor blood cells" refers to cells that are capable of producing antibodies when immunized with an antigenic compound. Examples of sources of such donor cells suitable for use in the invention include, but are not limited to spleen cells, lymph node cells, bone marrow cells, and immortalizing tumor infiltrating lymphocytes.

[0184] As used herein, the term "amino acid" denotes a molecule containing both an amino group and a carboxyl group. In some preferred embodiments, the amino acids are α-, β-, γ- or δ-amino acids, including their stereoisomers and racemates. As used herein the term "L-amino acid" denotes an α-amino acid having the L configuration around the α-carbon, that is, a carboxylic acid of general formula $CH(COOH)(NH_2)$-(side chain), having the L-configuration. The term "D-amino acid" similarly denotes a carboxylic acid of general formula $CH(COOH)(NH_2)$-(side chain), having the D-configuration around the α-carbon. Side chains of L-amino acids include naturally occurring and non-naturally occurring moieties. Non-naturally occurring (i.e., unnatural) amino acid side chains are moieties that are used in place of naturally occurring amino acid side chains in, for example, amino acid analogs. See, for example, Lehninger, BIOCHEMISTRY, Second Edition, Worth Publishers, Inc., 1975, pages 72-77 (incorporated herein by reference). Amino acid substituents may be attached through their carbonyl groups through the oxygen or carbonyl carbon thereof, or through their amino groups, or through functionalities residing on their sidechain portions.

[0185] As used herein "polynucleotide" refers to a nucleic acid molecule and includes genomic DNA cDNA, RNA, mRNA and the like.

[0186] As used herein "inhibitor of mismatch repair" refers to an agent that interferes with at least one function of the mismatch repair system of a cell and thereby renders the cell more susceptible to mutation.

[0187] As used herein "hypermutable" refers to a state in which a cell *in vitro* or *in vivo* is made more susceptible to mutation through a loss or impairment of the mismatch repair system.

[0188] As used herein "agents," "chemicals," and "inhibitors" when used in connection with inhibition of MMR refers to chemicals, oligonucleotides, RNA interference molecules, analogs of natural substrates, and the like that interfere with normal function of MMR.

[0189] As used herein, "about" refers to an amount within a range of +/- 10% of the cited value.

[0190] As used herein, "mitogenic polypeptide" refers to a polypeptide when in combination with the antigen provides stimulation of appropriate cells to increase the immune response against the subject antigen.

[0191] As used herein, "hybridoma" refers to the result of a cell fusion between an immunoglobulin-producing cell and a transformed cell, such as a myeloma cell.

[0192] As used herein, "IgG subclass" refers to a category of immunoglobulins comprising IgG1, IgG2, IgG2a, IgG2b, IgG3, and IgG4.

[0193] As used herein, "mismatch repair gene" refers to a gene that encodes one of the proteins of the mismatch repair complex. Although not wanting to be bound by any particular theory of mechanism of action, a mismatch repair complex is believed to detect distortions of the DNA helix resulting from non-complementary pairing of nucleotide bases. The non-complementary base on the newer DNA strand is excised, and the excised base is replaced with the appropriate base which is complementary to the older DNA strand. In this way, cells eliminate many mutations that occur as a result of mistakes in DNA replication. Dominant negative alleles cause a mismatch repair defective phenotype even in the presence of a wild-type allele in the same cell. A non-limiting example of a dominant negative allele of a mismatch repair gene is the human gene *hPMS2-134*, which carries a truncation mutation at codon 134. The mutation causes the product of this gene to abnormally terminateat the position of the 134th amino acid, resulting in a shortened polypeptide containing the N-terminal 133 amino acids. Such a mutation causes an increase in the rate of mutations which accumulate in cells after DNA replication. Thus, expression of a dominant negative allele of a mismatch repair gene results in impairment of mismatch repair activity, even in the presence of the wild-type allele.

[0194] As used herein, "HAT-sensitive" refers to a lethal effect on cells when cultured in medium containing hypoxanthine, aminopterin and thymidine.

[0195] As used herein, "EBV-negative" refers to lack of infection of Epstein-Barr virus in a cell as measured by production of EBNA protein, or detection of EBV nucleic acids.

[0196] As used herein, "Ig-negative" refers to lack of production in a cell of any light or heavy chains of immunoglobulins.

[0197] As used herein, "screening" refers to an assay to assess the genotype or phenotype of a cell or cell product including, but not limited to nucleic acid sequence, protein sequence, protein function (*e.g.*, binding, enzymatic activity, blocking activity, cross-blocking activity, neutralization activity, and the like). The assays include ELISA-based assays, Biacore analysis, and the like.

[0198] As used herein, "isolated" refers to a nucleic acid or protein that has been separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, the nucleic acid or protein is purified to greater than 95% by weight of protein. In other embodiments, the nucleic acid or protein is purified to greater than 99% by weight of protein. Determination of protein purity may be by any means known in the art such as the Lowry method, by SDS-PAGE under reducing or non-reducing conditions using a stain such as a Coomassie blue or silver stain. Purification of nucleic acid may be assessed by any known method, including, but not limited to spectroscopy, agarose or polyacrylamide separation with fluorescent or chemical staining such as methylene blue, for example.

[0199] The invention provides an *in vitro* immunization method for obtaining antigen-specific immunoglobulin producing cells wherein the cells produce immunoglobulins of the IgG subclass, and cells produced by this method. The *in vitro* immunization procedure comprises combining donor cells with an immunogenic antigen in culture. In one embodiment, the buffy coat of donor cells is used. The donor may be from any source, including, but not limited to cord blood, venous blood, and the like. The source of the blood cells may be from any animal producing immune cells, particularly mammals. Non-limiting examples of blood cell sources include, mice, rats, humans, monkeys, dogs, cats, horses, pigs, sheep, goats, rabbits, birds, cows, guinea pigs and fish. The blood or buffy coat may be further enriched for lymphocytes by any known method, such as, but not limited to differential centrifugation, filtration, and the like.

[0200] Donor cells such as peripheral blood mononuclear cells (PBMC) may be incubated in L-leucyl-L-lysine methyl ester hydrobromide (LLOMe). While not wishing to be bound by any particular theory of operation, LLOme is believed to lysosomotropic and specifically kills cytotoxic cells in the PBMC pool such as NK cells, cytotoxic T cells, and CD8+ suppressor T cells, while not having an effect on B cells, T helper cells accessory cells and fibroblasts (Borrebaeck (1988) *Immunol. Today* 9(11):355-359). Generally, the PBMCs may be

incubated with LLOMe for a period of 1-30 minutes. In some embodiments, the incubation is performed for 10-20 minutes. In other embodiments, the incubation is performed for 15 minutes. The LLOMe is generally a component of culture medium, such as, for example, RPMI 1640, and is provided in a concentration of about 0.10 to 1mM. In some embodiments, LLOMe is provided in an amount of about 0.10 to 0.50 mM. In other embodiments, LLOMe is provided in an amount of about 0.25 mM.

[0201] The antigen may be any antigen provided that it is immunogenic. Whole proteins or peptides may be used. In addition, one may use, for example, membrane preparations (including those from tumors), lymphoma cells, whole cells, single cells, homogenized cells, pathogens, inclusion bodies, cell lysates, protein preparations, and minced tissue (including tumor tissue). Whole proteins may be in native or denatured conformation. Peptides may be conjugated to carrier molecules to provide immunogenicity. While not wishing to be bound by any particular theory of operation, carrier molecules may provide additional T cell epitopes which may be useful in stimulating a more robust *in vitro* antibody response. Examples of carriers that are suitable for use in the method of the invention include tetanus toxoid, diptheria toxin, thyroglobulin, cholera toxin, BCG, bovine serum albumen (BSA), ovalbumin (OVA), and the like. These carriers are referred to herein as "mitogenic polypeptides."

[0202] Antigens may be conjugated to mitogenic polypeptides in any way known in the art. For example, fusion proteins may be generated by expressing a polypeptide in a recombinant expression system comprising the polynucleotide encoding at least a portion of the antigen joined in-frame to a polynucleotide encoding at least a portion of the mitogenic polypeptide. The fusion protein may have the mitogenic polypeptide joined at either the amino- or carboxy terminus of the antigen. In some embodiments, more that one antigen may be expressed as a fusion protein in combination with a mitogenic polypeptide. In other embodiments, more than one mitogenic polypeptide may be expressed as a fusion protein with the antigen or antigens. In other embodiments, more than one mitigenic polypeptide and more than one antigen may be expressed together as a single fusion protein.

[0203] In an alternative embodiment, at least a portion of the mitogenic polypeptide is conjugated to at least a portion of the antigen using chemical cross-linkers. Examples of chemical cross-linkers include, but are not limited to gluteraldehyde, formaldehyde, 1,1-bis (diazoacetyl)-2-phenylethane, N-hydroxysuccinimide esters (*e.g.*, esters with 4-azidosalicylic acid, homobifunctional imidoesters including disuccinimidyl esters such as 3,3'-dithiobis (succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane). Derivatizing agents such as methyl-3-[(p-azido-phenyl)dithio] propioimidate yield

photoactivatable intermediates which are capable of forming cross-links in the presence of light. Alternatively, for example, a lysine residue in the mitogenic polypeptide or antigen may be coupled to a C-terminal or other cysteine residue in the antigen or mitogenic polypeptide, respectively, by treatment with N-γ-maleimidobutyryloxy-succinimide (Kitagawa and Aikawa (1976) *J. Biochem.* 79, 233-236). Alternatively, a lysine residue in the mitogenic polypeptide or antigen may be conjugated to a glutamic or aspartic acid residue in the antigen or mitogenic polypeptide, respectively, using isobutylchloroformate (Thorell and De Larson (1978) RADIOIMMUNOASSAY AND RELATED TECHNIQUES: METHODOLOGY AND CLINICAL APPLICATIONS, p. 288). Other coupling reactions and reagents have been described in the literature.

[0204] The conditions for the *in vitro* immunization procedure comprise incubating the cells at about 25-37°C, (preferably 37°C) supplied with about 5-10% CO_2. In some embodiments, the incubation is performed with between about 6-9% CO_2. In other embodiments the incubation is performed in about 8% CO_2. The cell density is between about 2.5 to 5 x 10^6 cells/ml in culture medium. In some embodiments, the culture medium is supplemented with about 2-20% FBS. In other embodiments, the culture medium is supplemented with about 5-15% FBS. In other embodiments, the culture medium is supplemented with about 7-12% FBS. In other embodiments, the culture medium is supplemented with about 10% FBS.

[0205] The *in vitro* stimulation culture medium is supplemented with cytokines to stimulate the cells and increase the immune response. In general IL-2 is supplied in the culture medium. However, other cytokines and additives may also be included to increase the immune response. Such cytokines and factors may include, for example, IL-4 and anti-CD40 antibodies.

[0206] The fusion of myeloma cells with the immunoglobulin-producing cells may be by any method known in the art for the creation of hybridoma cells. These methods include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, *Nature* 256:495-497; and U.S. Patent No. 4,376,110) (see also, Brown *et al.* (1981) *J. Immunol.* 127:539-546; Brown *et al.* (1980) *J. Biol. Chem.* 255 (11):4980-4983; Yeh *et al.* (1976) *Proc. Natl. Acad. Sci. USA* 76:2927-2931; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-275), the human B-cell hybridoma technique (Kosbor *et al.*, 1983, *Immunology Today* 4:72; Cole *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80:2026-2030), and the EBV-hybridoma technique (Cole *et al.*, 1985, MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc., pp. 77-96). The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*.

[0207] The technology for producing monoclonal antibody hybridomas is well-known to those of skill in the art and is described, for example in Kenneth, in MONOCLONAL ANTIBODIES: A NEW DIMENSION IN BIOLOGICAL ANALYSES, Plenum Publishing Corp., New York, N.Y. (1980); Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; Galfre *et al.* (1977) *Nature* 266:55052; and Gefter *et al.* (1977) *Somatic Cell Genet.* 3:231-236). However, many variations of such methods are possible and would be appreciated by one of skill in the art. Thus, the techniques for generation of hybridomas is not limited to the disclosures of these references.

[0208] Any myeloma cell may be used in the method of the invention. Preferably, the myeloma cells are human cells, but the invention is not limited thereto or thereby. In some embodiments, the cells are sensitive to medium containing hypoxanthine, aminopterin, an thymidine (HAT medium). In some embodiments, the myeloma cells do not express immunoglobulin genes. In some embodiments the myeloma cells are negative for Epstein-Barr virus (EBV) infection. In preferred embodiments, the myeloma cells are HAT-sensitive, EBV negative and Ig expression negative. Any suitable myeloma may be used. An example of such a myeloma is that described in U.S. Patent No. 4,720,459 to Winkelhake, and deposited with the American Type Culture Collection (ATCC) as CRL 8644. Murine hybridomas may be generated using mouse myeloma cell lines (*e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines). These murine myeloma lines are available from the ATCC.

[0209] In some embodiments of the method of the invention, the hybridoma cells and/or mammalian expression cells may be rendered hypermutable by the introduction of a dominant negative allele of a mismatch repair gene. The dominant negative allele of the mismatch repair gene may be introduced into the hybridoma cell (*i.e.*, after the fusion of immunoglobulin-producing cells with the myeloma cells) or may be introduced into the myeloma cell prior to the fusions. The invention, therefore, also provides hypermutable myeloma cells for use in the generation of hybridoma cells. The dominant negative allele may also be introduced into the mammalian expression cells.

[0210] The dominant negative allele of the mismatch repair gene is in the form of a polynucleotide which may be in the form of genomic DNA, cDNA, RNA, or a chemically synthesized polynucleotide. The polynucleotide can be cloned into an expression vector containing a constitutively active promoter segment (such as, but not limited to, CMV, SV40, EF-1 Dor LTR sequences) or to inducible promoter sequences such as those from tetracycline, or ecdysone/glucocorticoid inducible vectors, where the expression of the dominant negative

mismatch repair gene can be regulated. The polynucleotide can be introduced into the cell by transfection.

[0211] Transfection is any process whereby a polynucleotide is introduced into a cell. The process of transfection can be carried out *in vitro*, *e.g.*, using a suspension of one or more isolated cells in culture. The cell can be any immortalized cell used for creating hybridomas for the production of monoclonal antibodies, or the cell may be the hybridoma itself. The hybridomas may be heterohybridoma cells (*e.g.* human-mouse cell fusions) or homohybridoma cells (*e.g.*, human-human hybridoma cells and mouse-mouse hybridoma cells).

[0212] In general, transfection will be carried out using a suspension of cells, or a single cell, but other methods can also be applied as long as a sufficient fraction of the treated cells or tissue incorporates the polynucleotide so as to allow transfected cells to be grown and utilized. The protein product of the polynucleotide may be transiently or stably expressed in the cell. Techniques for transfection are well known. Available techniques for introducing polynucleotides include but are not limited to electroporation, transduction, cell fusion, the use of calcium chloride, and packaging of the polynucleotide together with lipid for fusion with the cells of interest. Once a cell has been transfected with the mismatch repair gene, the cell can be grown and reproduced in culture. If the transfection is stable, such that the gene is expressed at a consistent level for many cell generations, then a cell line results.

[0213] The dominant negative allele of the mismatch repair gene may be derived from any known mismatch repair gene including, but not limited to *PMS2*, *PMS1*, *PMSR3*, *PMSR2*, *PMSR6*, *MLH1*, *GTBP*, *MSH3*, *MSH2*, *MLH3*, or *MSH1*, and homologs of *PMSR* genes as described in Nicolaides *et al.* (1995) *Genomics* 30:195-206 and Horii *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:1257-1264 and the like. "Dominant negative alleles" as used herein, refers to the ability of the allele to confer a hypermutable status to the cell expressing the allele. Any allele which produces such effect can be used in this invention. The dominant negative alleles of a mismatch repair gene can be obtained from the cells of humans, animals, yeast, bacteria, or other organisms. Dominant negative alleles of mismatch repair genes that are suitable for use in the invention have certain functional characteristics associated with structural features. A non-limiting example of a dominant negative mismatch repair gene is the PMS2 truncation mutant, PMS2-134. This gene contains a mutation which truncates the PMS2 protein after amino acid 133. The lack of the C-terminus in the PMS2 protein is believed to interfere with the binding of PMS2 with Screening cells for defective mismatch repair activity can identify such alleles. Cells from animals or humans with cancer can be

screened for defective mismatch repair. Cells from colon cancer patients may be particularly useful. Genomic DNA, cDNA, or mRNA from any cell encoding a mismatch repair protein can be analyzed for variations from the wild type sequence. Dominant negative alleles of a mismatch repair gene can also be created artificially, for example, by producing variants of the hPMS2-134 allele or other mismatch repair genes. Various techniques of site-directed mutagenesis can be used. The suitability of such alleles, whether natural or artificial, for use in generating hypermutable cells or animals can be evaluated by testing the mismatch repair activity caused by the allele in the presence of one or more wild-type alleles, to determine if it is a dominant negative allele.

[0214] Dominant negative alleles of such genes, when introduced into cells or transgenic animals, increase the rate of spontaneous mutations by reducing the effectiveness of DNA repair and thereby render the cells or animals hypermutable. This means that the spontaneous mutation rate of such cells or animals is elevated compared to cells or animals without such alleles. The degree of elevation of the spontaneous mutation rate can be at least 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, or 1000-fold that of the normal cell or animal. The hypermutable hybridoma cells will accumulate new mutations in gene(s) to produce new output traits within the hybridoma. The hybridoma cells can be screened for desired characteristics and cell lines bearing these characteristics may be expanded. Furthermore, the hybridoma cells may be "cured" of the mismatch repair defect by eliminating the dominant negative mismatch repair gene in the cell or by turning of its expression, leading to stable biological products consisting of altered genes, RNAs, or polypeptides.

[0215] The dominant negative alleles of the mismatch repair gene may be introduced as part of a vector. The polynucleotide encoding the dominant negative mismatch repair protein allele may be operably linked to a promoter that functions in the cell to drive expression of the dominant negative allele of the mismatch repair gene. Other elements of the vector may include an origin of replication, one or more selectable markers, such as a drug resistance gene that allows the cells to grow in the presence of a growth inhibitory compound.

[0216] In embodiments of the invention that utilize myeloma cells or donor immunoglobulin-producing cells that are naturally deficient in mismatch repair, the invention may further comprise the step of restoring genetic stability of the hybridoma by introducing a wild-type mismatch repair gene into the cell to complement the deficiency and restore genetic stability.

[0217] Another aspect of the invention is the use of cells lacking MMR (eitherdue to defects in endogenous mismatch repair genes, or due to the introduction of a dominant negative MMR gene) and chemical mutagens to cause an enhanced rate of mutations in a host's genome. The

lack of MMR activity has been known to make cells more resistant to the toxic effects of DNA damaging agents. This invention comprises making proficient MMR cells mismatch repair defective via the expression of a dominant negative MMR gene allele and then enhancing the genomic hypermutability with the use of a DNA mutagen. Chemical mutagens are classifiable by chemical properties, *e.g.*, alkylating agents, cross-linking agents, *etc.* The following chemical mutagens are useful, as are others not listed here, according to the invention and may be used to further enhance the rate of mutation in any of the embodiments of the method of the invention: N-ethyl-N-nitrosourea (ENU), N-methyl-N-nitrosourea (MNU), procarbazine hydrochloride, chlorambucil, cyclophosphamide, methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), diethyl sulfate, acrylamide monomer, triethylene melamin (TEM), melphalan, nitrogen mustard, vincristine, dimethylnitrosamine, N-methyl-N'-nitro-nitrosoguanidine (MNNG), 7,12 dimethylbenz (a) anthracene (DMBA), ethylene oxide, hexamethylphosphoramide, bisulfan. In a preferred aspect of the invention, a mutagenesis technique is employed that confers a mutation rate in the range of 1 mutation out of every 100 genes; 1 mutation per 1,000 genes. The use of such combination (MMR deficiency and chemical mutagens will allow for the generation of a wide array of genome alterations (such as but not limited to expansions or deletions of DNA segments within the context ofa gene's coding region, a gene's intronic regions, or 5'or 3' proximal and/or distal regions, point mutations, altered repetitive sequences) that are preferentially induced by each particular agent.

[0218] Mutations can be detected by analyzing for alterations in the genotype of the cells or animals, for example by examining the sequence of genomic DNA, cDNA, messenger RNA, or amino acids associated with the gene of interest. Mutations can also be detected by screening the phenotype of the gene. An altered phenotype can be detected by identifying alterations in electrophoretic mobility, spectroscopic properties, or other physical or structural characteristics of a protein encoded by a mutant gene. One can also screen for altered function of the protein *in situ*, in isolated form, or in model systems. One can screen for alteration of any property of the cell or animal associated with the function of the gene of interest, such as but not limited to measuring protein secretion, chemical-resistance, pathogen resistance,*etc.*

[0219] In some embodiments of the method of the invention, inducible vectors that control the expression of a dominant negative and normally functioning MMR gene are used. This strategy restores DNA stability once a host cell or organism exhibiting a new output trait, altered gene, RNA or polypeptide has been generated via trait selection with or without the combination of chemical mutagens to establish a genetically stable version of this cell or

organism. In the case of MMR defective cells as a result of ectopically expressing a dominant negative MMR gene allele, the MMR activity is decreased or completely eliminated by removing the inducer molecule from the cell culture or organism's environment. In addition, the expression of a dominant negative MMR gene can be suppressed by knocking out the MMR gene allele using methods that are standard to those skilled in the art of DNA knockout technology in germ or somatic cells (Waldman *et al.* (1995) *Cancer Res.* 55:5187-5190).

[0220] The chiral position of the side chains of the anthracenes is not particularly limited and may be any chiral position and any chiral analog. The anthracenes may also comprise a stereoisomeric form of the anthracenes and include any isomeric analog.

[0221] Examples of hosts are but not limited to cells or whole organisms from human, primate, mammal, rodent, plant, fish, reptiles, amphibians, insects, fungi, yeast or microbes of prokaryotic origin.

[0222] A more detailed disclosure of particular embodiments of the invention follows in the specific examples, however, the invention is not limited thereto or thereby.

EXAMPLES

Example 1: Generation of Hybridomas Secreting Human Monoclonal Antibodies to Tetanus Toxin (TT)

A. Generation and Assaying of TT-specific B lymphocytes

[0223] Isolation of lymphocytes from Donor. Lymphocytes were isolated from whole blood by centrifugation through Ficoll-Paque according to the manufacturer's instructions. Isolated lymphocytes were incubated with 0.25mM Leu-Leu methyl ester hydrobromide (LLOMe) prepared in RPMI 1640 medium containing 2% fetal bovine serum (FBS) for 15 minutes at room temperature. The cells were then washed three times with culture medium.

[0224] ***In vitro* stimulation of isolated lymphocytes.** The cells were incubated at 37°C in a incubator, supplied with 8% CO_2, at a density between 2.5 to 5 x 10^6 cells/ml in culture medium supplemented with 10% FBS and TT and IL-2 at various concentrations. After four days of culture, the cells were washed four times with medium and the culture was continued for additional eight days.

[0225] Measurement of the B cell response. Lymphocyte culture supernatants were collected on day 12 of the culture and tested in an ELISA for the presence of anti-TT antibodies. Briefly, TT or BSA at 0.5 μg/ml in 0.05 M carbonate-bicarbonate buffer was immobilized onto an EIA plate. After blocking with 1% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20, the supernatant was added to the wells. Antibodies bound to TT

were detected with peroxidase-labeled goat anti-human IgG or anti-human IgM. TMB was used for color development. The plate was read using a Microplate reader with a 450 nm filter. A supernatant sample that had antibody bound to TT, but not to BSA, and in which the signal was two times the assay background was considered positive. The positive cells were pooled, and used for hybridoma production (**Fig. 1**).

[0226] Notably, peripheral blood mononuclear cells (PBMCs) from some donors contain a fraction of B cells that secret TT-specific antibodies in culture. This is due to the fact that about 90% of the population in the United States has been vaccinated against TT. Such sera also has a titer of higher than 1000 (**Fig. 2**). However, the percentage of positive events is greatly increased when PBMCs are immunized *in vitro* with TT (**Fig. 3**). The intensity of the PBMC response is also enhanced with the stimulation of TT alone or in combination with IL-2 or CD40L (**Fig. 4**). Similar effects were observed with other antigens (data not shown).

[0227] Generation of hybridomas secreting human antibodies. To prepare activated lymphocytes, cells were pooled and cultured in T flasks at 0.5 –1 x 10^6 cells/ml in culture medium supplemented with 10% FBS one day prior to the fusion. To prepare the fusion partner, mouse myeloma NS0 cells were transfected with human PMS2-134 expression vector as described in Nicolaides *et al.* (1998) *Mol. Cell. Biol.* 18(3):1635-1641. The cells were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM glutamine (Complete Medium) and the culture was kept in log phase.

[0228] Next, lymphocytes were harvested and counted. An equal number of myeloma cells was harvested. Both types of cells were combined and washed three times withRPMI 1640 medium. Polyethylene glycol (PEG) was added dropwise to the loosened cell pellet, and the PEG was subsequently diluted out slowly with 25 ml of RPMI medium in a course of 2.5 minutes. After diluting out the PEG, fused cells were suspended in Complete Medium supplemented with HAT and 20% FBS, and seeded onto 96-well plates.

[0229] Screening and characterization of antigen-specific hybridoma clones. When the hybridoma cells grew to semi-confluence, supernatants were collected and subjected to an ELISA for antigen-specific reactivity. As an example, hybridomas derived from TT-immunized lymphocytes were tested. Briefly, TT or BSA at 0.5ug/ml in 0.05 M carbonate-bicarbonate buffer was immobilized onto the EIA plate. After blocking with 1% bovine serum albumen in PBS containing 0.05% Tween 20, the cell culture supernate was added to the wells. Antibodies bound to TT were detected with peroxidase-labeled goat anti-human IgG or anti-human IgM. TMB was used for color development. The plate was read in the Microplate reader with a 450 nm filter. A cell clone that showed reactivity to TT but not to BSA was

considered positive (**Fig. 5**). Positive clones were expanded and subcloned by limiting dilution to generate monoclonal cells.

Example 2: Generation of hybridomas secreting human monoclonal antibodies to epidermal growth factor receptor (EGFR) (self antigen)

A. Generation of EGFR-specific B lymphocytes

[0230] Preparation of Antigen. Human epidermal growth factor receptor (EGFR), purified from A431 cells, was purchased from Sigma. Previous studies found that immune responses to this antigen were very weak, most likely due to tolerance. In order to enhance immunization, we conjugated the EGFR to tetanus toxin C (EGFR-TT) and the conjugate was used as immunogen for *in vitro* immunization in order to overcome any immunotolerance.

[0231] Preparation of EGFR-TT conjugate. 100 ug of purified EGFR was reconstituted in 100 ul of sterile MilliQ-grade water. 1 mg of purified, lyophilized recombinant tetanus toxin C fragment (TT-C) was dissolved in sterile MilliQ-grade water to yield a 2 mg/ml TT-C solution. Crosslinking was performed in 50 mM sodium carbonate buffer pH 9.0 at equimolar ratios of EGFR to TT-C, using glutaraldehyde at a final concentration of 0.5% for 3 hours at room temperature, followed by 4°C overnight. Glutaraldehyde was quenched by addition of a fresh 100 mg/ml solution of sodium borohydride in 50 mM sodium carbonate pH 9.0, under open atmosphere for 1 hour at 4°C. Crosslinked products were dialyzed against Ca^{2+}, Mg^{2+}-free phosphate-buffered saline overnight at 4°C, using 3.5K MWCO Slide-A-Lyzer cassettes. The reaction was monitored by Western blotting, using commercial anti-EGFR (mAb-15) and anti-TT-C (Roche) monoclonal antibodies. By this method, greater than 70% of the components are crosslinked, and appear as immunoreactive species of greater MW than the starting material (data not shown).

[0232] *In vitro* stimulation of peripheral blood mononuclear cells (PBMC). LLOMe-pretreated PBMC were incubated at a density of 3 x 10^6 cells/ml in culture medium supplemented with 10% FBS and a stimuli mixture. The stimuli mixture was composed of EGFR-TT at a concentration of 50 ng/ml with or without recombinant human IL-2 at 20 IU, mouse anti-human CD40 antibody as CD40L at 0.5 ug/ml (used to enhance IgG class switching). After four days of culture, the cells were re-fed with complete medium, in the absence of added stimulus, every three or four days. Culture supernatants were collected on days 12-18 and tested for EGFR-specific antibodies.

[0233] Detection of EGFR-specific antibody response. The PBMC response to the stimulation was examined in a EGFR-specific ELISA. Briefly, EGFR, TT, or BSA at 0.5 ug/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6, was immobilized onto EIA plates.

After blocking the plates with 5% non-fat dry milk in PBS containing 0.05% Tween 20, the supernatant was added to the wells. Antibodies from the supernatant bound to immobilized antigens were detected with peroxidase-labeled goat anti-human IgG+IgM (H+L). TMB substrate kit was used for color development. The plates were read in a Microplate reader with a 450 nm filter. A supernatant sample containing antibody that bound to EGFR, but not to TT and BSA, was considered positive. A robust response was observed in cultures immunized to the EGFR-TT as compared to controls. While anti-EGFR responses were observed in PBMCs for a small fraction of donors, the percentage of positive clones was greatly increased when PBMCs were immunized *in vitro* with EGFR complexed with TT (**Fig. 6**). Positive cells were pooled and used for hybridoma production.

[0234] Generation of Hybridomas. To prepare activated lymphocytes, cells were pooled and cultured in T flasks at $0.5 - 1 \times 10^6$ cells/ml in culture medium supplemented with 10% FBS one day prior to the fusion. To prepare the fusion partner, mouse myeloma NS0 cells were transfected with human PMS2-134 expression vector as described in Nicolaides *et al.* (1998) *Mol. Cell. Biol.* 18(3):1635-1641. The cells were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM glutamine (Complete Medium) and the culture was kept in log phase.

[0235] Screening and characterization of antigen-specific hybridoma clones. When the hybridoma cells grew to semi-confluence, supernatants were collected and subjected to an ELISA for antigen-specific reactivity. As an example, hybridomas derived from EGFR-immunized lymphocytes were tested. Briefly, EGFR, TT TNFR1 orBSA at 0.5 ug/ml in 0.05 M carbonate-bicarbonate buffer was immobilized onto the EIA plate. After blocking with 1% bovine serum albumen in PBS containing 0.05% Tween 20, the cell culture supernate was added to the wells. Antibodies bound were detected with peroxidase-labeled goat anti-human IgG or anti-human IgM. TMB was used for color development. Normal human IgG (nhIgG) and IgM (nhIgM) were used as controls. The plate was read in the Microplate reader with a 450 nm filter. A cell clone that showed reactivity to EGFR, but not to BSA was considered positive (**Fig. 7**).

Example 3

A. Isolation of PBMC from whole blood

[0236] Approximately 200ml of whole blood mixed with 200ml $PBS^{-/-}$ was centrifuged through Ficoll-Paque at 2000rpm for 30min. Serum was aspirated, the interface layer containing lymphocytes was collected and diluted 1:3 with $PBS^{-/-}$ and centrifuged at 2000 rpm for 10min. The supernatant fluid was aspirated and the pellet was resuspended in 10 ml

$PBS^{-/-}$. The cell suspension was split into two 50 ml conical tubes and $PBS^{-/-}$ was added to each tube to adjust the volume to 35 ml each. The tubes were centrifuged at 800 rpm for 7 minutes to remove the platelets. After aspirating the supernatant fluid, the pellet was resuspended in 10 ml ACK Lysing Buffer and incubated for 5 minutes at room temperature. Following lysis, 35 ml $PBS^{-/-}$ was added to the tubes and the tubes were centrifuged at 1000 rpm for 7 minutes. The cells were then washed with 45 ml RPMI medium.

B. Preparation of Dendritic Cells

[0237] Cells were centrifuged at 1000 rpm for 7 minutes and resuspended at 1 x 10^8 cells per 40 ml cRPMI for a density of 2.5 x 10^6 cells/ml. The cells were incubated at 37°C/8% CO_2 for 2 hours. Non-adherent cells were removed for further treatment (see Step C), and the adherent cells were carefully rinsed twice with $PBS^{-/-}$. Adherent cells were cultured in cRPMI supplemented with 400 IU/ml IL-4 and 50 ng/ml GM-CSF.

C. LLOMe treatment and cryopreservation of non-adherent culture

[0238] The non-adherent cell culture was centrifuged at 1000 rpm for 7 minutes. The supernatant fluid was aspirated and the pellet was resuspended in 10 ml RPMI supplemented with 2% FBS and freshly thawed 85 µg/ml LLOMe. The cells were incubated for 15 minutes at room temperature. The cells were washed twice with cRPMI and resuspended in 45 ml cRPMI. The cells were transferred to an upright T25 flask at a density of 5 x 10^6 cells/ml in cRPMI supplemented with 2 µg/ml PHA and incubated at 37°C/8% CO_2 for 24 hours. The non-adherent cells were harvested, centrifuged at 1000 rpm for 7 minutes, and the cell pellet was resuspended in 5 ml cold cRPMI/5%DMSO. The tubes containing the cells were wrapped in paper towels and stored at -80°C until needed.

D. Tumor Immunization

[0239] On day 6 of the procedure for isolation of dendritic cells, tumor cells were thawed in 2.5 ml pre-warmed medium at 37°C. The flask of dendritic cells was rinsed twice with 10 ml $PBS^{-/-}$. The dendritic cells were incubated with gentle rocking in 5 ml Cell Dissociation Buffer (Invitrogen Cat. No. 13151-014), and the solution was collected (scraping the remaining cells from the flask. The flask was rinsed with 10 ml cRPMI and the medium was collected. The cells were centrifuged at 1000 rpm for 7 minutes and the pellet was resuspended at 4 x 10^6 cells/ml cRPMI. Cells were distributed in a culture plate at a density of 1 x 10^6 cells/well. A tumor sample was chopped into fine pieces of approximately 1-3 mm^3.

An aliquot of the tumor suspension was transferred to all but 1 well, titrating the amount of tumor per well. An aliquot of 0.25 ml cRPMI was added to the control well. The total volume in the wells was 0.5ml/well. The dendritic cells and tumor cells were co-cultured at 37°C/8% CO_2 for 24 hours.

E. Co-culture of PBMC with DC

[0240] Frozen PBMC were thawed by adding 40 ml cRPMI/30 IU/ml IL-2/600 IU/ml IL-4/0.75 µg/ml CD-40L pre-warmed to 50°C to the frozen cells. When thawed, the cells were incubated for 1-2 hours at 37°C. The cells were centrifuged at 1000 rpm for 7 minutes and the pellet was resuspended in 5 ml of a 2X cocktail of cRPMI/ 60 IU/ml IL-2/1200 IU/ml IL-4/1.5µg/ml CD-40L. The cell suspension was divided among wells in a tissue culture plate at 0.5 ml/well of suspension and diluted with 0.5 ml medium for a final concentration of 30 IU/ml IL-2, 600 IU/ml IL-4, and 0.75 µg/ml CD-40L. Cells were fed with cRPMI supplemented with 20 IU/ml IL-2, 400 IU/ml IL-4, 100 IU/ml IL-10, and 0.5µg/ml CD-40L.

F. Fusion

[0241] Tumor-immunized PBMCs were then fused with A6 myeloma cells to generate hybridomas. Briefly, lymphocytes were harvested from 75% tumor and 100% tumor wells, rinsed with 1 ml RPMI, transfer to conical tubes, and the volume was adjusted to 5 ml with cRPMI. The cells were centrifuged through Ficoll-Paque, and the supernatant fluid was aspirated. Interfaces containing cells from all tubes were combined and the cells were rinsed with cRPMI. The cells were then resuspended in 7.5 ml cRPMI. Viable cells were assessed by trypan blue exclusion. A6 cell viability was also assessed by trypan blue exclusion. A6 cells and tumor-immunized lymphocytes were centrifuged separately at 1200 rpm for 10minutes. The supernatant fluids were aspirated and the cells were washed with 10ml DPBS $^{-/-}$/tube. Each cell line was washed three times with 2 ml cold Mannitol Fusion Medium (MFM) (0.3M Mannitol, 0.18mM $MgCl_2$, 0.18mM $CaCl_2$, 1mM Hepes)and the cells were combined and resuspended in MFM at a density of 3 x 10^6 A6 cells and 3 x 10^6 PBMCs in 200 µl for a total of 6 x 10^6 cells in 200 µl. BTX 450 microslides were sterilized with 65µL 100% EtOH and pre-wetted with 65µl MFM. A 40µl aliquot of cell suspension was distributed evenly onto a BTX 450-1 microslide. To fuse the cells, the ECM 2001 conditions were set as follows: alignment conditions, 20V for 30 seconds; pulse conditions, 150V for 30 µseconds (1X); compression conditions, 20V for 9 seconds. After fusion, the cells were transferred to one well of a 24 well plate containing 1 ml phenol red-free cRPMI. The fusion steps were

repeated for the remaining cell suspensions, rinsing slide between fusions with 65 µL MFM. The culture plate containing fused cell cultures was incubated overnight at 37°C/8%CO_2. The fused cells were cloned and assessed by ELISA for IgG and IgM production. The results are shown in **Fig. 8**.

Example 4: *In vitro* immunization

Purified GM-CSF from a commercial source is administered *in vitro* to peripheral blood mononuclear cells (PBMC).

A. Generation and Assaying of GM-CSF-specific B lymphocytes

Isolation of lymphocytes from Peripheral Blood.

[0242] Lymphocytes are isolated from whole blood by centrifugation through Ficoll-Paque according to the manufacturer's instructions. Isolated lymphocytes are incubated with 0.25 mM Leu-Leu methyl ester hydrobromide (LLOMe) prepared in RPMI 1640 medium containing 2% fetal bovine serum (FBS) for 15 minutes at room temperature. The cells are then washed three times with culture medium.

***In vitro* stimulation of isolated lymphocytes.**

[0243] The cells are incubated at 37°C in an incubator, supplied with 8% CO_2, at a density between 2.5 to 5 x 10^6 cells/ml in culture medium supplemented with 10% FBS and GM-CSF and IL-2 at various concentrations. After four days of culture, the cells are washed four times with medium and the culture was continued for additional eight days.

Measurement of the B cell response.

[0244] Lymphocyte culture supernatants are collected on day 12 of the culture and tested in an ELISA for the presence of anti-GM-CSF antibodies. Briefly, GM-CSF or BSA at 0.5 µg/ml in 0.05 M carbonate-bicarbonate buffer is immobilized onto an EIA plate. After blocking with 1% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20, the supernatant is added to the wells. Antibodies bound to GM-CSF are detected with peroxidase-labeled goat anti-human IgG or anti-human IgM. TMB is used for color development. The plate is read using a Microplate reader with a 450 nm filter. A supernatant sample that had antibody bound

to GM-CSF, but not to BSA, and in which the signal was two times the assay background is considered positive. The positive cells are pooled, and used for hybridoma production.

Generation of hybridomas secreting human antibodies.

[0245] To prepare activated lymphocytes, cells are pooled and cultured in T flasks at 05 – 1 x 10^6 cells/ml in culture medium supplemented with 10% FBS one day prior to the fusion. To prepare the fusion partner, mouse myeloma NS0 cells are transfected with human PMS2-134 expression vector as described in Nicolaides *et al.* (1998)*Mol. Cell. Biol.* 18(3):1635-1641. The cells are cultured in RPMI 1640 supplemented with 10% FBS and 2 mM glutamine (Complete Medium) and the culture is kept in log phase.

[0246] Next, lymphocytes are harvested and counted. An equal number of myeloma cells is harvested. Both types of cells are combined and washed three times with RPMI 1640 medium. Polyethylene glycol (PEG) is added dropwise to the loosened cell pellet, and the PEG is subsequently diluted out slowly with 25 ml of RPMI medium in a course of 2.5 minutes. After diluting out the PEG, fused cells are suspended in Complete Medium supplemented with HAT and 20% FBS, and seeded onto 96-well plates.

Screening and characterization of antigen-specific hybridoma clones.

[0247] When the hybridoma cells grew to semi-confluence, supernatants are collected and subjected to an ELISA for antigen-specific reactivity. As an example, hybridomas derived from TT-immunized lymphocytes are tested. Briefly, TT or BSA at 0.5 ug/ml in 0.05 M carbonate-bicarbonate buffer is immobilized onto the EIA plate. After blocking with 1% bovine serum albumin in PBS containing 0.05% Tween 20, the cell culture supernate is added to the wells. Antibodies bound to GM-CSF are detected with peroxidase-labeled goat anti-human IgG or anti-human IgM. TMB is used for color development. The plate is read in the Microplate reader with a 450 nm filter. A cell clone that showed reactivity to GM-CSF but not to BSA is considered positive. Positive clones are expanded and subcloned by limiting dilution to generate monoclonal cells.

Example 5: Generation of hybridomas secreting human monoclonal antibodies to GM-CSF-KLH

A. Generation of GM-CSF-specific B lymphocytes

Preparation of Antigen.

[0248] Human GM-CSF was purchased from a vendor. In order to enhance immunization, GM-CSF was conjugated to keyhole limpet hemocyanin (KLH) (GM-CSF-KLH) and the conjugate was used as immunogen for *in vitro* immunization in order to overcome any immunotolerance.

Preparation of GM-CSF-KLH conjugate.

[0249] Purified GM-CSF was reconstituted in sterile MilliQ-grade water to yield a 1mg/ml solution. Purified, lyophilized recombinant KLH was dissolved in sterile MilliQ-grade water to yield a 1 mg/ml KLH solution. A 0.2% solution of glutaraldehyde in PBS was prepared. Crosslinking was performed by combining 25 ul of 1 mg/ml KLH, 25 ul of 1 mg/ml GM-CSF, and 50 ul 0.2% glutaraldehyde in a microcentrifuge tube wrapped in aluminum foil at room temperature, with shaking for 1 hour. Following cross-linking, 25 ul of 1 M glycine was added to the tube and the solution was incubated an additional 1 hour at room temperature with shaking. Crosslinked products were dialyzed against three changes of 300 ml PBS. The reaction was monitored by Western blotting, using a commercial anti-GM-CSF and anti-KLH monoclonal antibodies. By this method, greater than 80% of the components are crosslinked, and appeared as immunoreactive species of greater MW than the starting material (data not shown).

***In vitro* stimulation of peripheral blood mononuclear cells (PBMC).**

[0250] LLOMe-pretreated PBMC were incubated at a density of 3 x 10^6 cells/ml in culture medium supplemented with 10% FBS and a stimuli mixture. The stimuli mixture was composed of GM-CSF-KLH at a concentration of 50 ng/ml with or without recombinant human IL-2 at 20 IU, mouse anti-human CD40 antibody as CD40L at 0.5 ug/ml (used to enhance IgG class switching). After four days of culture, the cells were re-fed with complete medium, in the absence of added stimulus, every three or four days. Culture supernatants were collected on days 12-18 and tested for GM-CSF-specific antibodies.

Detection of GM-CSF-specific antibody response.

[0251] The PBMC response to the stimulation was examined in a GM-CSF-specific ELISA. Briefly, GM-CSF, KLH, or chick ovalbumin (CAB) at 0.5 ug/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6, was immobilized onto EIA plates. After blocking the plates with 1% BSA containing 0.05% Tween 20, the supernatant were added to the wells. Antibodies from the supernatant bound to immobilized antigens were detected with peroxidase-labeled goat anti-human IgG+IgM (H+L). TMB substrate kit was used for color development. The plates were read in a Microplate reader with a 450 nm filter. A supernatant sample containing antibody that bound to GM-CSF, but not to KLH and CAB, was considered positive. There was a robust response observed in cultures immunized to the GM-CSF-KLH as compared to controls. While anti-GM-CSF responses were observed in PBMCs for a small fraction of donors, the percentage of positive clones was greatly increased when PBMC were immunized *in vitro* with GM-CSF complexed with KLH. Positive cells were pooled and used for hybridoma production.

Generation of Hybridomas.

[0252] To prepare activated lymphocytes, cells were pooled and cultured in T flasks at 0.5 – 1 x 10^6 cells/ml in culture medium supplemented with 10% FBS one day prior to the fusion. To prepare the fusion partner, mouse myeloma NS0 cells were transfected with human PMS2-134 expression vector as described in Nicolaides *et al.* (1998)*Mol. Cell. Biol.* 18(3):1635-1641. The cells were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM glutamine (Complete Medium) and the culture was kept in log phase.

Screening and characterization of antigen-specific hybridoma clones.

[0253] When the hybridoma cells grew to semi-confluence, supernatants were collected and subjected to an ELISA for antigen-specific reactivity. As an example, hybridomas derived from GM-CSF-immunized lymphocytes were tested. Briefly, GM-CSF, KLH, or CAB at 0.5 ug/ml in 0.05 M carbonate-bicarbonate buffer was immobilized onto the EIA plate. After blocking with 1% bovine serum albumin in PBS containing 0.05% Tween 20, the cell culture supernate was added to the wells. Antibodies bound were detected with peroxidase-labeled goat anti-human IgG or anti-human IgM. TMB was used for color development. Normal human IgG (nhIgG) and IgM (nhIgM) were used as controls. The plate is read in the

Microplate reader with a 450 nm filter. A cell clone that showed reactivity to GM-CSF, but not to CAB was considered positive. The results are shown in **Fig. 9**

Example 6

Inhibition of Proliferation Assays

[0254] TF-1 cells were seeded at 0.2 x 10^6/ml in RPMI supplemented with 10% FBS and 0.5 ng/ml recombinant human GM-CSF. TF-1 cells were serum starved for 24 hours in medium containing 0.5% BSA, without rhGM-CSF. Cells were then cultured in the presence of 0.275ng/ml of GM-CSF for 3 days, with or without 4 ug/ml of various antibodies. Cell proliferation was measured using the ATPLite assay (Perkin Elmer). In this assay, ATP was released by lysis of viable cells and utilized by the enzyme luciferase to convert luciferin into oxyluciferin. Light was emitted (luminescence) as a result of the reaction, and the intensity of the emission was ultimately proportional to the ATP content and thus to the cell number. Counts per second (CPS) were obtained by reading the reactions with a luminometer and the percentage of inhibition was calculated according to the formula: 100 - (CPS no Ab : CPS with Ab) x 100%. The results are shown in **Fig. 10**.

[0255] The foregoing examples are merely illustrative of the invention and are not to be construed to limit the scope of the invention in any way. The scope of the invention is defined by the appended claims.